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GROWTH FACTOR LOADED-MICROPARTICLES AS A TOOL FOR CARDIAC REPAIR

Trabajo presentado por **Fabio Rocha Formiga** para obtener el Grado de Doctor

Fdo. Fabio Rocha Formiga

Licenciado en Farmacia

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CERTIFICAN

Que el presente trabajo: "*Growth factor loaded-microparticles as a tool for cardiac repair",* presentado por **Fabio Rocha Formiga** para aspirar al grado de Doctor, ha sido realizado bajo su dirección en el Departamento de Farmacia y Tecnología Farmacéutica de la Universidad de Navarra en colaboración con el Departamento de Área de Terapia Celular de la Clínica Universidad de Navarra y del Centro de Investigación Médica Aplicada (CIMA) y, una vez revisado, no encuentra objeciones para que sea presentado a su lectura y defensa.

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Ang	Angiopoietin
BSA	Bovine serum albumin
C-GSF	Colony granulocyte stimulating factor
CHD	Coronary heart disease
CHF	Chronic heart failure
cMLCK	Cardiac-specific myosin light-chain kinase
cTnT	Cardiac troponin T
CVD	Cardiovascular diseases
DMEM	Dubelcco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
E/A	Peak E and A transmitral filling velocity ratio
EC	Endothelial cell
ECM	Extracellular matrix
EGF	Epidermal growth factor
EPC	Endothelial progenitor cell
EPO	Erythropoietin
ErbB	NRG tyrosine kinase receptor
FDA	U.S. Food and drug administration
FGF-1	Acidic fibroblast growth factor
FGF-2	Basic fibroblast growth factor
FGFR	FGF tyrosine kinase receptor
G-CSF	Granulocyte colony-stimulating factor
GDNF	Glial cell-line derived neurotrophic factor
GF	Growth factor
HE	Hematoxylin–eosin
HGF	Hepatic growth factor
HIAEC	Human iliac artery endothelial cell line
HIF-1α	Hypoxia inducible factor-1α
HSA	Human serum albumin
HSPGs	Heparan sulfate proteoglycans
IHD	Ischemic heart disease

IL-1β	Interleukin-1β
IL-6	Interleukin-6
IMCs	Inflammation-mediated cells
LAD	Left anterior descending
LV	Left ventricle
LVEDD	Left ventricular end-diastolic diameter
LVEDV	Left ventricular end-diastolic volume
LVEF	Left ventricle ejection fraction
LVESD	Left ventricular end-systolic diameter
LVESV	Left ventricular end-systolic volume
MCP-1	Monocyte chemoattractant protein-1
MI	Myocardial infarction
MMPs	Matrix metalloproteinases
MP	Microparticles
M_w	Molecular weight
NL	Non-loaded
NO	Nitric oxide
NRG	Neuregulin
PBS	Phosphate-buffered saline
PCADK	poly(cyclohexane-1,4diyl acetone dimethylene ketal)
PDGF	Platelet-derived growth factor
PDGFR	PDGF tyrosine kinase receptor
PEG	Poly(ethylene glycol)
PEO	Poly(ethylene oxide)
PLGA	poly-lactide-*co*-glycolide
PVA	poly(vinyl alcohol)
rh	Recombinant human
SEM	Scanning electron microscopy
Shh	Sonic hedgehog
TGF- β	Transforming growth factor-β
Tie	Ang tyrosine kinase receptor

TNF-α	Tumor necrosis factor-α
TROMS	Total recirculation one-machine system
TUNEL	Terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling
VEGF	Vascular endothelial growth factor
VEGFR	VEGF tyrosine kinase receptor
VSMC	Vascular smooth muscle cell
W_1	Inner aqueous phase
$W_1/O/W_2$	Multiple emulsion
W_2	Outer aqueous phase
WHF	World heart federation
WHO	World health organization
α-SMA	Alpha smooth muscle actin

INTRODUCTION

INTRODUCTION

Angiogenic therapy for cardiac repair based on protein delivery systems

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ABSTRACT

Cardiovascular diseases remain the first cause of morbidity and mortality in the developed countries and are a major problem not only in the western nations but also in developing countries. Current standard approaches for treating patients with ischemic heart disease include angioplasty or bypass surgery. However, a large number of patients cannot be treated using these procedures. Novel curative approaches under investigation include gene, cell and protein therapy. This review focuses on potential growth factors for cardiac repair. The role of these growth factors in the angiogenic process and the therapeutic implications are reviewed. Issues including aspects of growth factor delivery are presented in relation to protein stability, dosage, routes and safety matters. Finally, different approaches for controlled growth factor delivery are discussed as novel protein delivery platforms for cardiac regeneration.

Keywords: Cardiovascular diseases, cardiac repair, growth factor, angiogenesis, protein delivery

1. INTRODUCTION

Cardiovascular diseases (CVD) are, globally considered, the main cause of death in the world. The concept of CVD includes several disorders of the heart and blood vessels, such as ischemia, rheumatic and inflammatory heart disease. Table 1 summarizes the World Health Organization (WHO) data regarding deaths from this cause, published in 2008 [1]. Ischemic heart disease (IHD) is the main problem within CVD and, according to The World Heart Federation (WHF) information, the number of deaths it causes every year is similar in Europe and in South-East Asia, revealing that CVD are a major problem all over the world. Moreover, the WHF report (2008) on the economic impact of diseases shows the high cost of treatment for CVD in developed countries, which in the United States (USA), for example, is as high as €310.23 billion: more than twice the cost of all cancers [2, 3].

IHD occurs when a coronary artery narrows, frequently as a result of atherosclerosis, and blood supply in the heart is insufficient, resulting in angina, heart attack, or even sudden death of the patient. When faced with ischemia, the heart tries to make up for the loss of functionality and cardiac remodeling starts. This process is responsible for important alterations in myocyte biology, as well as for myocardial changes, alterations in extracellular matrix (ECM) and in the left ventricular chamber geometry. Briefly, after ischemia, changes at the level of the failing human cardiac myocyte lead to a defect in contractile function. On the other hand, myocardium itself fails as a consequence of myocyte loss through both necrotic and apoptotic cell death, perivascular fibrosis around intramyocardial blood vessels and excessive deposition of fibrillar collagen around myocytes. These changes affect the ventricular chamber geometry, involving the emergence of a larger and a more spherical heart shape. The

combination of all these anatomic, functional and biological alterations contributes to progression of the disease [4] as described in Fig. 1.

Current therapies include pharmacological treatments, percutaneous intervention and surgery. However, although these can mitigate the symptoms, they are not able to regenerate the tissue, or to restoring the heart function. Furthermore, for a number of patients, the only alternative is organ transplantation, with all its drawbacks. This has moved researchers and clinicians to explore new approaches.

Table 1. Deaths (000s) by cause in WHO Regions, adapted from: Estimates for 2004. (The global burden of disease: 2004 update. WHO)

Region / **Cause**	Africa	The Americas	Eastern Mediterranean	Europe	South-East Asia	Western Pacific	World
I. Communicable diseases, maternal and perinatal conditions and nutritional deficiencies	7,682	835	1,664	567	5,636	1,568	17,971 (30.6%)
II. Non-communicable conditions	2,797	4,737	2,157	8,137	7,695	9,428	35,017(59.6%)
• Malignant neoplasms	480	1,180	296	1,862	1,195	2,398	7,424(12.6%)
• Cardiovascular diseases	1,175	1,969	1,163	4,767	3,875	4,094	17,073(29%)
- Ischemic heart diseases	346	925	579	2,296	2,011	1,029	7,198(12.2%)
III. Injuries	769	586	485	789	1,949	1,196	5,784 (9.8%)
TOTAL DEATHS	11,248	6,158	4,306	9,493	15,279	12,191	58,772 (100%)

Among others, these have focused on restoring blood flow by inducing angiogenesis by treatment with cells, genes or soluble factors involved in this process.

This review examines proposed options for the treatment of cardiovascular diseases based on the induction of tissue revascularization, particularly focusing on protein-based therapy and the use of controlled drug delivery systems.

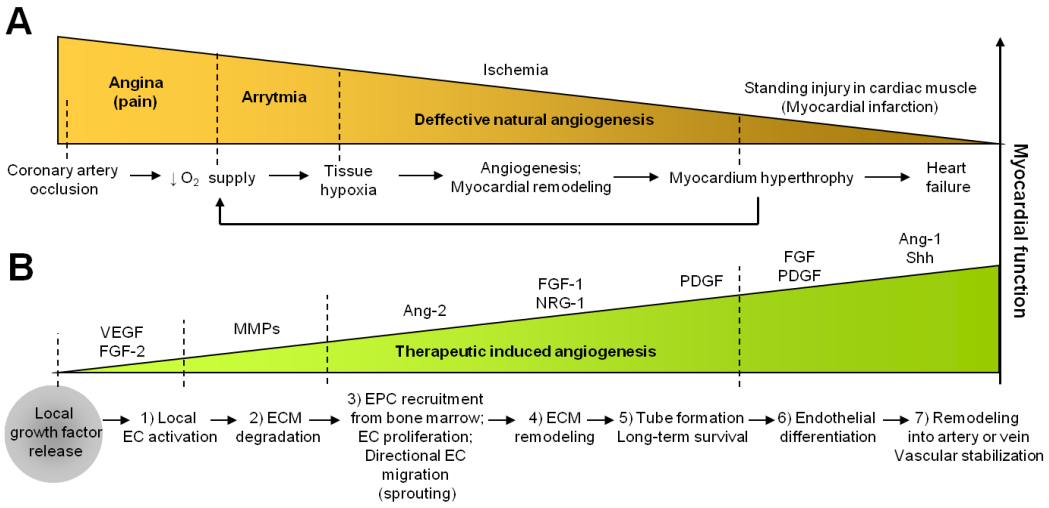

Fig. 1. The playground for therapeutic angiogenesis: A) When a coronary occlusion happens, the oxygen local supply decreases dramatically and the tissue responds to hypoxia by inducing transcription of proangiogenic factors, cytokines and matrix metalloproteinases (MMPs). The myocardium attempts to restore oxygen supply and replace the damage tissue. However, often these adaptative responses are not effective and myocardium hypertrophy occurs. Thereafter, there is a permanent injury which would lead to heart failure. B) If a local controlled release of angiogenic factor/s such as FGFs (Fibroblast Growth Factors), VEGF-A (Vascular Endothelial Growth Factor-A), Ang (Angiopoietin), PDGF (Platelet-derived Growth Factor), etc. is carried out following heart injury, the endogenous process of angiogenesis and remodeling would be enhanced over time, allowing effective revascularization, and recovery of myocardial function could ultimately be achieved (EC, endothelial cell; ECM, extracellular matrix; EPC, endothelial progenitor cell).

2. THERAPEUTIC ANGIOGENESIS

Angiogenesis is the process of formation of new vascular vessels from the existing ones, by sprouting and longitudinal division (intussusception) processes. It also involves incorporation of endothelial progenitors recruited from the bone marrow (postnatal

vasculogenesis). The newly formed vessels split and branch into pre-capillary arterioles and capillaries.

Angiogenesis is a crucial phenomenon during embryonic development, but it also occurs in adult tissues under certain physiological circumstances: ovulation, development of the corpus luteum, immune response, inflammation and wound repair. This natural means of giving rise to new vessels is a complex process involving different types of cells, secreted soluble factors (with pro- and anti-angiogenic activities) and extracellular matrix compounds, which operate in a tightly regulated spatial and temporal manner. The outcome (adequate, defective or excessive angiogenesis) depends on the balance between angiogenic activators and inhibitors, and their imbalance may result in pathology because of either excessive or insufficient angiogenesis (Fig. 2). In such cases, several pathologies (brain, cardiac or peripheral ischemia, defective healing in diabetes, etc.) could benefit from therapeutic induction of angiogenesis.

In protein-based therapeutic angiogenesis, one or various exogenous proteins are administered to intervene in the endogenous process at several levels: reducing inflammatory response, controlling ECM renovation, and promoting survival, proliferation, differentiation and migration of cells. The induced therapeutic cardiac environment allows sprouting, branching and maturation of new vessels into arteries and/or veins. In this way, metabolic homeostasis and contractile function would be restored and the recovery of cardiac function could ultimately be achieved.

3. POTENTIAL FACTORS FOR THERAPEUTIC MYOCARDIAL ANGIOGENESIS

Tumor research led to the finding of factors responsible for angiogenesis and their applications as therapy for some ischemic diseases such as myocardial ischemia [5]. Along similar lines, the more recent knowledge acquired about the factors involved in cardiovascular development during embryogenesis has led researchers to translate these factors to promote cardiac repair in the adult organism. Nowadays it is known that proangiogenic factors expressed in the embryo are newly induced in the adult heart under hypoxia and stress conditions to achieve revascularization when the coronary artery flow is disrupted [6].

Below are described several of the main proangiogenic factors which would be suitable for its use in therapeutic angiogenesis, indicating their signaling pathways, their biological actions and the relationships between them.

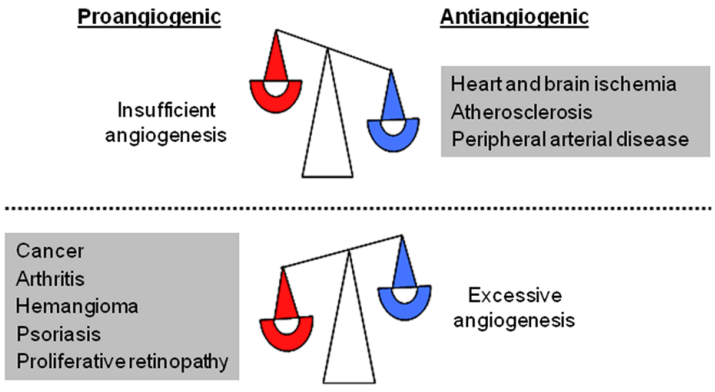

Fig. 2. Consequences of the imbalance in the angiogenesis process.

3.1. Fibroblast Growth Factor (FGF)

FGF was one of the first angiogenic growth factors related to tumor vascularization to be discovered [7, 8]. The FGF family comprises one of the more versatile growth

factor signaling systems in vertebrates, acting in a wide variety of biological process. In mice and humans, twenty three FGF ligands and four tyrosine kinase receptors (FGFR), which are subjected to multiple splicing events, have been identified [9]. FGF-1 (acidic FGF) and FGF-2 (basic FGF) are the most extensively studied members and to date, are the only FGFs known that are involved in cardiac repair.

At the myocardium, FGFs are pleiotropic molecules that act on ECs, smooth muscle cell and myoblasts, which express high-affinity FGF receptors. The binding of FGF ligand to FGFR leads to the dimerization and autophosphorilation of the receptor and this event triggers, either directly or through the recruitment of adaptor proteins, the activation of several intracellular signaling pathways that result in different cellular responses involved in angiogenesis and cardiac repair. Among them, several functions have been described, such as the induction of 1) proliferation of ECs, smooth muscle cells and myoblasts [10]; 2) survival of cardiomyocytes, vascular smooth muscle cells (VSMCs) and ECs (reviewed in [11]); 3) cell-cell interactions and physical organization of ECs into tube-like structures [12]; 4) VEGF secretion in endothelial and stromal cells (autocrine mechanism of FGF induced angiogenic response) [13, 14]; 5) induction of PDGF receptor expression in VSMCs (contributing to maturation-stabilization of newly formed vessels) [15] and 6) selective upregulation of MCP-1 (monocyte chemoattractant protein-1) on non-endothelial mesenchymal cells (VSMCs and fibroblasts) (contributing to the arteriogenesis driven by immune cells) [16].

3.2. Vascular Endothelial Growth Factor (VEGF)

VEGF was discovered as a factor that induces vascular hyperpermeability and acts as an endothelial cell-specific mitogen [17]. Since then, VEGF has been the protein

most widely used to induce angiogenesis both in pre-clinical models and in clinical assays.

In humans, the VEGF family currently comprises members encoded by five genes: VEGF-A (the first identified as VEGF), -B, -C (also called VEGF-2), -D and PlGF (Placental Growth Factor). Due to alternative splicing, multiple isoforms with different biological activities can be produced from each gene. Active VEGFs are mainly homodimers, although VEGF-A and PlGF heterodimers have also been identified. VEGFs present different extracellular distribution and each isoform can bind to co-receptors (neuropilins) or ECM compounds, namely heparin and/or heparan sulfate proteoglycans (HSPGs) [18].

VEGFs are implicated in the vascular development during embryogenesis and in new blood vessel formation in the adult [19]. VEGF-A is the best characterized member and it shows the highest angiogenic potential. Several human VEGF-A isoforms have been identified: VEGF-$A_{145,}$ VEGF-A_{189}, and VEGF-A_{206} which are bound tightly to cell surface; VEGF-A_{121}, a highly diffusible form; VEGF-A_{165a} and VEGF-A_{165b}, which exist as both bound and freely diffusible protein [20].

VEGFs can bind to three receptor tyrosine kinases, known as VEGFR-1 (Flt-1), VEGFR-2 (KDR/Flk-1) and VEGFR-3 (Flt-4). Although highly homologous, they exhibit different affinities for the VEGF ligands. VEGFR-1 and VEGFR-2 are expressed predominantly by vascular ECs to participate in vascular angiogenesis while VEGFR-3 in adult is mainly confined to the lymphatic endothelium. VEGFR-1 has a higher affinity for VEGF-A, but it has a much weaker kinase activity and is unable to generate a mitotic response in ECs. In contrast, VEGFR-2 has a lower affinity for VEGF-A but it is able to signal and hence trigger multiple cell responses. VEGFR-1 can

also exist in soluble form, binding to VEGF without any signaling, and thus limiting the availability of VEGF-A to VEGFR-2 (reviewed in [21]).

After VEGF ligand binding, VEGFR goes through dimerization and autophosphorilation, triggering the recruitment of cytoplasmic interacting proteins and activation of several signaling molecular pathways involved in a variety of responses in ECs like: 1) permeability [22]; 2) survival [23]; 3) proliferation [24] and 4) migration (reviewed in [18]).

The angiogenic effect of VEGF-A is regulated at different levels (reviewed in [20]). Firstly the expression of VEGF-A can be induced by several stimuli such as HIF-1α (for its part is up-regulated by FGF-2), growth factors (PDGF-BB, FGF-4, Transforming Growth Factor-β or TGF- β) and inflammatory cytokines (Interleukins-1 or 6, Tumor Necrosis Factor-α or TNF- α, etc.). Secondly, the duration and intensity of VEGFR signaling can be modulated by co-receptors such as HSPGs and neuropilins, and also through interaction with adhesion molecules regulated by blood flow. Ultimately transcription of VEGFR-2 is also induced by HIF-1α and TNF-α. Furthermore the interaction between endothelial and smooth muscle cells can also regulate VEGF signal (read below how other factors secreted by these cells affect the VEGF response).

3.3. Angiopoietins (Ang)

This family of growth factors consists of four members of secreted glycoproteins named Ang-1, Ang-2, Ang-3 and Ang-4. The ones which are best known for their involvement in cardiovascular remodeling are Ang-1 and Ang-2. These two members show some differences which could account for the outcome of their signaling. Both bind to tyrosine kinase receptor Tie-2 on ECs with similar affinity, but they act in an

opposite way. While the binding of Ang-1 to Tie-2 promotes its autophosphorylation and the subsequent intracellular signaling, Ang-2 acts as a natural antagonist since it binds to Tie-2 without the autophosphorylation event. This may be due to differences in the structure of the domain responsible for receptor binding. Another important feature is that Ang-1 is produced by non-ECs in many tissues and it is incorporated into the ECM, while Ang-2 is accumulated or secreted in a soluble form by ECs in sites of vascular remodeling. This could regulate their availability and biological activity [25]. Moreover, the outcome of angiopoietin signaling depends on the balance between Ang-1 and Ang-2. In fact, during cardiovascular development Ang-1 is expressed early and Ang-2 is detected later [26].

The Ang-1 signaling induces multiple effects on ECs: chemotaxis, tube formation and survival inhibiting endothelial apoptosis through several intracellular pathways. However, there is no evidence of endothelial proliferation in response to Ang-1 [27]. It has also been shown that Ang-1 is able to oppose the permeability action of VEGF-A, inducing the recruitment of pericytes and smooth muscle cells to be incorporated in the vessel wall, besides anti-inflammatory actions. So Ang-1 may have a leading role in vessel maturation and stabilization, regulating cell-cell and cell-matrix interactions [21, 28].

On the other hand, the binding of Ang-2 to Tie-2 avoids Ang-1 signaling, leading to vessel destabilization, activation of ECs to respond to angiogenic stimuli (such as VEGF), detachment of pericytes and degradation of ECM. In this way, Ang-2 allows the subsequent sprouting initiated by VEGF. *In vitro* [29] and *in vivo* [30] evidence suggests that under low oxygen tension Ang-2 could act in a biphasic way, initially blocking Ang-1 signaling and allowing ECs stimulation by angiogenic factors and next,

contributing to the stabilization and maturation of the newly formed blood vessels. Some studies have shown Ang-2 up regulation and Ang-1 down regulation mediated by hypoxia [31]. Moreover, there is evidence for a coordinated relationship between VEGF and Ang-2 levels. At low levels of VEGF, Ang-2 signaling leads to vascular regression, but in the presence of higher level of VEGF the outcome of Ang-2 signaling is sprouting and vessel formation [21, 32].

3.4. Platelet-derived Growth Factor (PDGF)

The first isoform of the PDGF family was discovered in the mid 1970s as a constituent of platelet α-granules with growth promoting activity for fibroblast and smooth muscle cells. Subsequently it has been shown that PDGF is produced in different isoforms by distinct cell types under normal and pathological scenes (during organogenesis, angiogenesis, tissue fibrosis, in tumors, etc). So far, four isoforms of PDGF ligands have been identified: PDGF-A, -B, and more recently -C and –D. These four polypeptides require proteolytic cleavage and dimerization to achieve biological activity. The active homo or heterodimers PDGF-AA, -BB, -AB, -CC and –DD bind to tyrosine-kinase receptors PDGFRs. There are two types of receptors, PDGFR-α and PDGFR-β, which can be expressed in a selective or dual manner depending on the cell type (i.e., PDGFR-β is expressed rather specifically on VSMCs and pericytes, whereas ECs in sprouting vessels express elevated levels of both –α and –β receptors [33]. PDGF-A binds specifically to PDGFR-α whereas PDGF-B can bind to PDGFR-α and PDGFR-β. PDGF-C and PDGF-D bind preferably to PDGFR-β but it seems they also can bind to PDGFR-α on cells expressing both α and β receptors).

The PDGF signal induces over 80 genes, among them matrix and cytoskeleton proteins, growth factors, growth inhibitors, transcription factors involved in cell cycle (c-jun, c-fos, c-myc), etc. One of the physiological functions of PDGF/PDGFR signal is to participate in angiogenesis and vessel stabilization through stimulation of proliferation and migration of vascular ECs, VSMCs, fibroblasts, monocytes and granulocytes (reviewed in [34]). Several studies have found that administration of PDGF-BB or -AB in combination with FGF-2, leads to an increase in capillary and arteriolar density and vessel stabilization, in models of hind limb ischemia and chronic myocardial infarction in rats [35, 36]. Recently, this effect has been attributed specifically to PDGFR-β, but not to PDGFR-α. A possible mechanism suggested for this angiogenic synergy and vascular stability is that FGF-2 induces a strong up regulation of PDFGR on endothelial cells, leading to formation of receptor dimers with persistent activity even after removal of PDGF ligands, that would maintain the angiogenic response [37].

Lately, more interest has been focused on PDGF-C, which presents a wide range of direct and indirect angiogenic effects [38], such as increasing the number and availability of ECs, pericytes and smooth muscle cells and the induction of proliferation of fibroblasts and inflammatory cells, therefore increasing production of angiogenic growth factors, ECM and matrix metalloproteinases that will allow the growth of new vessels and remodeling of arterioles into arteries [39].

3.5. Neuregulin-1 (NRG-1)

To date, NRG-1 is the only neuregulin known to be involved in the development and function of the heart [40]. It presents three distinct isoforms that arise from gene

transcription from different promoters: type I, type II and type III. All of them are synthesized as membrane-anchored precursors, and type I and type II NRG-1 are solvable by proteolytic processing signaling to nearby cells in a paracrine manner. On the other hand, mature type III NRGs remains anchored and signals to adjacent cells in a juxtacrine manner [41].

NRG-1 is a member of EGF (Epidermal Growth Factor) family and structurally consists of four main domains. The extracellular EGF-like domain gives rises, by alternative splicing, to α and β isoforms. These isoforms differ in their binding ability, since the β isoforms exhibit 10-100 more activity when binding to receptor.

In spite of the variability, all NRG isoforms perform their biological activity through the tyrosine-kinase ErbB membrane receptors. It appears that during heart development only the type I and type II NRG-1β isoforms have a critical role, but in the adult heart type I NRG-1α is the one predominantly expressed although the NRG-1β isoform continues to be important. NRG-1 ligands appear to be produced on ECs near cardiomyocytes (in the myocardial microvasculature and endocardium) in response to oxidative stress in adult heart [42]. Related to the ErbB receptors, ErbB-2, ErbB-3 and ErbB-4 are critical for heart development, the ErbB3 expression being lost in adult cardiomyocytes [43].

In cardiomyocytes, the NRG-1 ligands bind to the ErbB-4 receptor which dimerizes with ErbB2, leading to multiple cellular responses like the proliferation and survival of neonatal [43] and adult cardiomyocytes [44-46]. Moreover, it has been shown that in pathological conditions, NRG-1 promotes myocardial regeneration and decreases hypertrophy of surrounding infarcted areas [47] by preserving a synchronized beat (through activation of the Src/FAK (Focal Adhesion Kinase) pathway (involved in

sarcomeric organization and cell-cell interactions) and upregulation of the cMLCK (a cardiac-specific myosin light-chain kinase that controls muscle contraction and sarcomere organization)) [42, 48]). Also, NRG1 is involved in the Ca^{2+} homeostasis (involved in myocyte relaxation [49]), the control of the inotropic response to adrenergic stimulation (due to stress or overload) [50] and indirect paracrine angiogenic effect on ECs, through the release of VEGF-A by other cell types (such as fibroblast) [51].

All of these effects have prompted the potential therapeutic use of NRG-1 in patients with heart disease. Recently, two clinical assays have been carried out in Australia and in China (later referred to in the section 4, [52] and [53]).

3.6. Sonic hedgehog (Shh)

Shh is a lipoprotein that belongs to the Hedgehog (Hh) family of morphogens. The Hh gene was discovered in a developmental study in Drosophila melanogaster [54], with three Hh homologues in vertebrates later being indentified: Desert (Dhh), Indian (Ihh) and Sonic Hedgehog (Shh) [55-57]. Among these, Shh shows the most widespread expression in embryo and in adult tissues with many important functions in the organism, including a crucial role during heart vasculature development (extensively reviewed in [58]) and tissue homeostasis, acting in repair processes after severe injury (tissue regeneration, tissue injury, ischemia and hypoxia, inflammation, etc.) (reviewed in [59]).

Shh is synthesized in the cytoplasm as a precursor protein which undergoes autocleavage and lipidation resulting in the active Shh form (ShhN, about 20 kDa), consisting of the N-terminal signaling domain (Shh-N) with a cholesterol moiety at the

carboxy-terminal and palmitoylation at the N terminus. These lipidic modifications of Shh take account of its distribution from the producing cell and it is thought to be involved in several mechanisms affecting the extent of the signaling [60, 61]. ShhN could thereby act either in long-range or in a short-range signaling (by cell-cell contact) resulting in paracrine or autocrine responses. During development, Shh acts mainly as a morphogen by long-range signaling, but in adult tissues the short range signal is most important during repair (reviewed in [62]).

The Shh protein activates several signaling pathways, a canonical one that acts through the Patched receptor that leads to activation and nuclear translocation of Gli transcription factors, which will drive the transcription of several angiogenic genes among others (reviewed in [59]), and a recently described "non-canonical" signaling cascade, which is transcription/translation-independent, and which activates leukotriene metabolism leading to reorganization in the cytoesqueleton to drive the migration towards the Shh-N source [63, 64].

Despite its complexity, some investigations in mice have elucidated the critical role of Shh signaling in the maintenance of adult coronary vasculature by promoting angiogenesis and cell survival [65]. Also, during myocardial repair after ischemia, Shh-N seems to be delivered by fibroblasts and acts on endothelium, VSMCs and cardiomyocytes. Like other angiogenic factors, it has been recently shown that hypoxia can trigger HIF-1α-mediated Shh expression, within as little as 1 hour [66], inducing vascular remodelling by nitric oxide (NO) production in ECs [67, 68], upregulation of anti-apoptotic molecules in cardiomyocytes [69], release of angiogenic factors (VEGF and Angiopoietins) by cardiac fibroblasts [70] and recruitment of bone marrow derived-EPCs [69]. Regarding the therapeutic potential, Shh protein or gene delivery approaches

have shown angiogenesis induction in myocardial ischemia models both in mice and rats [69-71]. Also, Shh has been shown to be a critical mediator of erythropoietin-induced cardiac protection [72]. However, the role of endogenous Shh-N is controversial as some data indicate the Hh signal can contribute to injury during myocardial ischemia [73].

4. CLINICAL TRIALS WITH PROTEIN THERAPY

Protein-based therapy has been explored in clinical settings for the promotion of angio- and arteriogenesis in the ischemic myocardium by delivering angiogenic growth factors. The clinical studies with recombinant proteins performed in patients suffering from IHD are listed in Table 2. In most of the trials, patients presented severe coronary artery disease, which could not be treated adequately with conventional revascularization therapies.

The first phase-I clinical trial was performed in 20 patients with three vessel disease [74]. In this study, FGF-1 was intramyocardially injected in patients undergoing coronary artery bypass of the left anterior descending coronary artery (LAD). In this study, safety was proven but, despite an increased capillary density, no evidence of coronary perfusion or ventricular function improvement was determined.

Also, parenteral administration of FGF-2 in humans was first tested in a small placebo-controlled, dose-escalation safety study performed in 25 patients with coronary artery disease and stable angina. In this study, 17 patients received intracoronary infusion of recombinant FGF-2 and 8 patients, placebo infusion. Few side-effects such as mild hypotension, slight transient trombocytopenia and proteinuria were registered but without further complications [75]. In another study, intracoronary infusions of

FGF-2 were also well tolerated in another study with 52 patients. In this case, patients were sub-optimal candidates for conventional revascularization. At the two-month follow-up, the patients presented fewer angina symptoms, improved exercise capacity and reduced ischemic territory. Dose-related hypotension was detected and four deaths and four major cardiac events occurred but did not appear to be related to dose or time of administration [76]. Taken together, the results of all phase I studies using FGF-2 suggested that intracoronary delivery of this growth factor was reasonably safe and may produce functionally significant clinical benefits. Next, a multi-center, randomized, double-bind, placebo-controlled phase-II trial (FIRST) with a single intracoronary infusion of recombinant FGF-2 at different doses (0.3, 3 and 30 μg/kg) was performed, but the results were disappointing. Although a significant reduction in clinical angina was detected in the 3 μg/kg group, no significant effect was detected at 180 days in any of the treated groups. In addition, single intracoronary infusion of FGF-2 did not improve exercise tolerance or myocardial perfusion [77].

On the other hand, the results of small phase I trials using intracoronary and intravenous infusions of VEGF-A in patients with coronary artery disease have been encouraging [78-80]. For example, Hendel et al. reported a significant improvement in exercise capacity without any safety issues. Also, the resting nuclear myocardial perfusion scans indicated a VEGF-A treatment effect [79]. However, a randomized, double-blind, placebo-controlled phase II trial of VEGF-A also failed to show differences between the treatment and placebo groups [81]. Another study, The VIVA, compared two doses of VEGF-A to placebo in 178 patients with coronary artery disease. A single intracoronary infusion followed by three separate intravenous infusions was given. Despite the safety and tolerability, the administration regimes

revealed that VEGF-A offered no improvement beyond placebo by day 60, although high-dose VEGF-A resulted in better improvement in angina and favorable trends in exercise treadmill test time and angina frequency, by day 120. Perhaps the most striking contribution of the VIVA trial was to consider that more preclinical data were needed with regard to the time course of angiogenesis and the optimal dose and route of administration to induce effective VEGF-A therapy in the myocardium.

In addition to studies using VEGF-A and FGF proteins, other growth factors known to have a role in tissue repair and angiogenesis have been tested in myocardial clinical settings, including colony granulocyte stimulating factor (C-GSF)[82-85], hepatocyte growth factor (HGF) [86], erythropoietin (EPO) [87, 88] and neuregulin. Regarding the latter, two human studies aimed at exploring the safety and efficacy of recombinant NRG-1 in chronic heart failure (CHF) have been recently performed. Jabbour et al. reported sustained haemodynamic effects, as demonstrated by the 12% increase in left ventricle ejection fraction (LVEF) at 12 weeks in patients treated with daily infusion of NRG-1 for 11 days [52]. The Chinese Phase II clinical trial using a short-term administration of rhNRG-1 in CHF patients could result in sustained improvement of cardiac pumping and ventricular anti remodeling compared with baseline, although these changes were not statistically significant between NRG-1 and the placebo groups [53].

In general, although the therapy was safe and well tolerated, statistically significant efficacy was not consistently demonstrated in the clinical trials involving angiogenic growth factors. However, as part of intensive research on protein-based therapy for cardiac repair, further clinical studies are now in progress in patients with coronary artery disease. A new FGF-1 delivery technique is being performed by means of the

Myostar® catheter (Cordis Corp., J&J company) in the CardioVascular BioTherapeutics phase II clinical trial (ClinicalTrials.gov Identifier: NCT00117936). Another ongoing phase II study involves the parenteral administration of EPO to evaluate the effect of this growth factor on damage to the heart in patients with acute heart attacks (ClinicalTrials.gov Identifier: NCT00378352).

As a conclusion to these studies, the results of myocardial clinical trials using protein delivery have generally been disappointing and the studies have failed to consistently demonstrate improvements in treated patients as compared with placebo. Many of these trials relied on an intravenous infusion or intracoronary delivery of the recombinant protein. Therefore these negative results have been attributed, at least partially, to the short lived effect and high instability of the protein when injected as a bolus. For example, from pharmacokinetic data collected from the FGF-1 studies in the human heart, it appears that FGF-1, once it exits the heart, is cleared from the circulation in less than three hours [89]. Intravenous administration of VEGF-A is limited by its short *in vivo* half life (~30 min) and overall dose is limited by off-target site toxicity issues [81, 90]. In the case of myocardial ischemia, the amount of VEGF-A localized in the ischemic region after systemic administration is minimal and does not persist for more than 1 day [91]. Indeed, the short permanence in the heart of the administered proteins after intracoronary delivery might be an important cause for the missing clinical effect [92].

Local and sustained combined growth factor delivery by controlled release approaches in the heart tissue might be a better strategy to achieve higher efficacy in protein-based therapy for myocardial ischemia. However, many issues remain to be established, such as protein formulation, stability, dosage, routes and safety.

Table 2. Myocardial vascularization clinical trials using recombinant proteins

Protein	Route	Trial	n	Primary Endpoint	Outcomes	Clinical Trial Identifier	Reference
FGF-1	IM	Phase I	20	Neoangiogenesis in angiography at 90 days	Increased capillary density, but no evidence of improved coronary perfusion or ventricular function		[74]
FGF-2	IC	Phase I	25	Safety monitoring and tolerability at 3 days	Dose-escalation trial; doses of 3 to 30 μg/kg was generally well tolerated in subjects with stable angina; no signs of systemic angiogenesis		[75]
	IC	Phase I	52	ETT at 29 days	Improvements in exercise tolerance and reduction in size of ischemic area		[76]
	IC/IV	Phase I	59	Improved myocardial perfusion at 29 days	Ascending dose trial; improvement in perfusion and attenuation of stress-induced ischemia; no control group		[93]
	IC	Phase II	337	ETT and angina frequency at 90 days	FIRST study; significant reduction in symptoms of angina at 90 days follow-up, but no longer detectable at 180 days; no improvement in ETT time and myocardial perfusion		[77]
VEGF-A	IC	Phase I	14	Improved myocardial perfusion at 30 days	Some improvement in perfusion in patients treated with low-dose rhVEGF-A; five of six patients had perfusion improvement on rest and stress at higher doses		[79]
	IC	Phase I	15	Improved myocardial perfusion at 60 days	Dose screening study; well tolerated up to 0.05 mg/kg/min; myocardial perfusion imaging was improved in 7 of 14 patients at 60 days		[80]
	IV	Phase I	28	Myocardial perfusion	Evidences of improvement in rest myocardial perfusion and in collateral density		[78]
	IC/IV	Phase II	178	ETT at 60 days	VIVA study; safe and well tolerated; no improvement beyond placebo in all measurements by day 60. By day 120, high-dose rhVEGF-A resulted in significant improvement in angina; no improvements in exercise tolerance; no improvements in myocardial perfusion		[81]

G-CSF	SC	Phase I	52	Coronary collateral flow and ECG at 14 days	Subcutaneous G-CSF is efficacious during a short-term protocol in improving signs of myocardial salvage by coronary collateral growth promotion	ClinicalTrials.gov NCT00596479	[82]
	SC	Phase II	60	LVEF at 180 days	Increased end-diastolic volume from baseline to 6 months in the placebo group but unchanged in the G-CSF group; no significant differences in LVEF or perfusion between groups		[83]
	SC	Phase III	100	Adverse events and compliance at 6 weeks	SITAGRAMI-Trial; combined application of G-CSF and Sitagliptin; planned first interim-analysis on safety issues: only two major adverse cardiac events occurred (one de novo stenosis and one in-stent-restenosis) in the first 36 patients	EudraCT Number 2007-003941-34	[85]
NRG	IV	Phase I	15	Haemodynamics at 2h and LVEF at 12 weeks	Acute and sustained improvements in cardiac function; safe and well tolerated; no control group	ACTRN12607000 330448	[52]
	IV	Phase II	44	LV function and structure at 90 days	Progressive improvement of cardiac function and anti remodeling effect in patients with chronic heart failure, but no statistically significant differences	ChiCTR-TRC-00000414	[53]
EPO	IV	Phase I	44	Erythropoietin activity; angiogenesis markers	Evidence of safety and biologic activity of erythropoietin in patients with acute myocardial infarction; increased expression of angiogenesis signaling proteins	ClinicalTrials.gov NCT00367991	[87]
	IV	Phase II	529	LVEF at 6 weeks	A single high dose of EPO did not improve LVEF after 6 weeks	ClinicalTrials.gov NCT00449488	[88]

FGF-1: acidic Fibroblast Growth Factor; FGF-2: basic Fibroblast Growth Factor; VEGF: Vascular Endothelial Growth Factor; G-CSF: Granulocyte colony-stimulating factor; NRG: Neuregulin; EPO: Erythropoietin; IM: Intramyocardial; IC: Intracoronary; IV: Intravenous; SC: Subcutaneous; LVEF: Left ventricle ejection fraction; ETT: exercise tolerance testing; ECG: electrocardiogram; ANZCTR: Australian New Zealand Clinical Trials Registry, http://www.anzctr.org.au; ChiCTR: Chinese Clinical Trial Registry, http://www.chictr.org/; EudraCT: European Clinical Trials Database, https://eudract.ema.europa.eu/

5. CHALLENGES IN PROANGIOGENIC FACTOR DELIVERY

Although protein growth factors that play essential roles in angiogenesis and arteriogenesis have been deeply studied, the suitable manner for making these cytokines available at the target site with a desired dosage and for a determined period of time remains unclear. Also, the ability to efficiently incorporate and release multiple angiogenic factors that mimic the natural microenvironment of the tissue needs to be determined.

5.1. Growth factor dosage and routes of administration

The limited success of the protein-based angiogenic therapy may be related partially to the way of growth factor delivery. As has been shown previously, several delivery routes have been tested in patients including intravenous, intracoronary, intramyocardial and perivascular administration (Fig. 3). Intravenous infusions are appealing because of their practicality, but have a minimal effect in producing angiogenesis [94]. Intracoronary delivery is easily performed with catheter-based techniques but may lead to low protein deposition into the myocardium. Detailed analysis of FGF-2 uptake and retention one hour after its injection showed that only 0.9% and 0.26% of the injected FGF-2 was found in the ischemic myocardium after intracoronary and intravenous administration, respectively. Still, only very low levels of the protein remained in the myocardium 24 hours later (0.05% for intracoronary and 0.04% for intravenous delivery) [95]. Also, intrapericardial administration cannot be used in post-cardiac surgery patients. Therefore, site-specific methods such as intramyocardial delivery are preferred since it includes the possibility of targeting the desired areas of the myocardium, and has a higher delivery efficiency and prolonged

tissue retention. Growth factors can be injected intramyocardially into the border zone of the infarct or the centre of the ischemic area. Alternatively, proteins can be intramyocardially targeted by endocardial injection with a specialized intraventricular catheter. Yet, epicardial zones can be targeted via thoracoscopy without the need for open-chest surgery.

The protein amount retained by the target tissue may be considered to establish a suitable dosage. Previously, the range of effective concentrations used for *in vitro* studies acted as an important guidance. Also, tissue condition (perfused or non-perfused areas) and route of administration may act as critical factors to determine protein concentration at the myocardium [96, 97]. Therefore, protein threshold dosage may be established based on previous *in vitro* assays and tissue distribution studies.

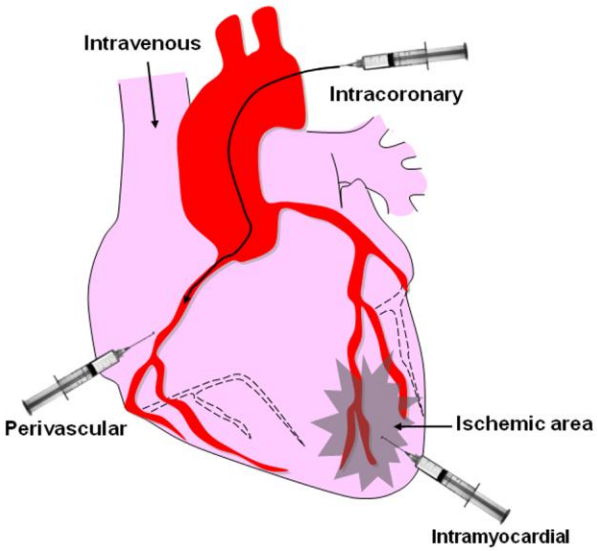

Fig. 3. Growth factor delivery to the myocardium. Proteins can be targeted to the myocardium by several routes and each one has both merits and drawbacks. Intravenous delivery is a practical strategy, but is not likely to produce functional angiogenesis in the target tissue; also, the downside includes systemic exposure to a growth factor and potential for unwanted effects such as hypotension. Intracoronary delivery can be performed using catheter-based techniques and may be effective when adequate doses are used, regarding the low protein deposition in the myocardium. Intramyocardial delivery may provide better myocardial distribution and retention than intracoronary and intravenous routes and, like perivascular delivery it can be performed either via open chest or via thoracoscopy.

5.2. Protein stability

Like protein-based compounds, growth factor molecules are not conventional drugs. A critical issue in protein formulation is the retention of biological activity, as well as the preservation of biological function at pharmacological concentrations for therapeutic effect. Safe, effective and reliable protein formulation requires an in-depth understanding of the properties of the protein, particularly its susceptibility to either chemical or physical instability. During pre-formulation research, protein stability should be assessed using a complementary set of well-established analytical techniques such as SDS-PAGE, circular dichroism, fluorescence, FTIR, dynamic light scattering, size exclusion chromatography, differential scanning calorimetry, etc. [98].

Since protein and peptide drugs are highly susceptible to proteolysis or rapidly cleared from the circulation or from the target site, it has been necessary to control the protein drug delivery. Thus, a critical step is to develop delivery platforms able to protect and release therapeutic proteins effectively. Recent years have witnessed significant progress for improvement and innovation in nano- and microparticles, hydrogels and scaffold manufacture, in order to deliver delicate macromolecules. Indeed, incorporation of therapeutic proteins into polymer devices has been a suitable strategy to protect these special drugs by adding excipients such as buffers, stabilizing sugars and amino acids, surfactants and protein carriers like albumin. These substances are useful in helping to prevent protein adsorption to surfaces, interfacial denaturation and aggregation [99, 100].

5.3. Safe angiogenesis

Therapeutic angiogenesis is not free from potential harmful effects. Despite the critical role of different growth factors in the physiological angiogenesis and survival of endothelial cells, there is considerable evidence that some cytokines are important tumor angiogenic factors [101, 102]. In general, high doses of recombinant proteins or prolonged exposure to the proteins may cause various side effects including tumor growth, but also hypotension, edema, proteinuria, hemorrhage, diabetic retinopathy, plaque rupture, and angioma formation. Thus, for example, unexpected side effects of FGF-2 therapy have been reported, indicating that protein dosage must be carefully monitored [103]. Careful control of proangiogenic molecules both in dosage and in localization is important to improve the local therapeutic efficiency of the protein and avoid unwanted side effects. Some of the toxic effects have been confirmed in animal models, but the limited results from clinical settings seem to refute some of the aforementioned risks or only show mild and transient effects. A larger number of clinical trials need to be conducted to clarify the possible undesired side effects.

6. CONTROLLED GROWTH FACTOR DELIVERY SYSTEMS

Regarding the issues mentioned above, multiple efforts have been made to overcome these limitations. In general, controlled drug delivery systems have many advantages over bolus or repetitive administration. Patient compliance, drug protection and sustained release are some of the many benefits of incorporating and releasing a therapeutic molecule from an adequate matrix (such as hydrogels, particles, scaffolds, capsules, etc.). Controlled release strategies have demonstrated the importance of maintaining precise concentrations of active GFs over days or weeks and orchestrating the timing of GF release proximal to the site of desired angiogenesis. Also, the matrix may emulate the highly functionalized role of ECM in modulating the stability, activity, release, and spatial localization of GFs [104].

6.1. Polymer-based growth factor delivery systems

Polymers can serve as a matrix for controlled drug delivery as some properties can be modified by changing the monomers ratio and composition, controlling polymerization conditions, or introducing functional groups to the polymers [105]. A number of approaches have been reported on the protein controlled release from polymeric matrices, such as nano- and microparticles, hydrogels, polymer scaffolds and other delivery devices by using natural and synthetic materials. Table 3 summarizes potential and currently used materials in which GFs can be incorporated to stimulate angiogenesis. Important approaches based on targeted GF delivery systems for cardiac repair in animal models of myocardial ischemia are also showed (Table 4).

Table 3. Natural and synthetic biomaterials used in angiogenic growth factor delivery

Biomaterial	Properties	Applications	References
	Naturals		
Collagen/ gelatin	Important component of ECMs and forms thermally reversible gels; functionally important qualities such as adhesiveness for cells and proteolytic degradability are retained in gelatin	Porous interconnecting network for EC adhesion and migration, and collagen hydrogel for angiogenic GF release in a controlled manner	[106-108]
Fibrin	Sealing malleable matrix prepared from autologous plasma and available as glue or as engineered microbeads	Fibrin-based hydrogels can be surgically applied as sealant and adhesive in fibrin glue (mixture of concentrated fibrinogen and thrombin usually derived by cryoprecipitation of human plasma): useful as GF-controlled release systems to stimulate angiogenesis	[104, 109-112]
Hyaluronic acid	Glycosaminoglycan present in the natural ECM and composed of repeating units of D-glucuronic acid and N-acetyl-D-glucosamine; HA forms hydrogels by various covalent cross-linking methods; high biocompatibility and biodegradability	Stimulation of *in vivo* angiogenesis by HA hydrogels loaded with GFs such as VEGF-A, bFGF and KGF	[113-115]
Alginate	Nontoxic polysaccharide-based polymer of marine origin with the fraction and sequence of the two monomers, α-L-guluronic and β-D-mannuronic acid sugar residues varying over a wide range; ECM-mimetic features, physical cross-linking, biocompatibility and erosion	Alginate microspheres, beads and hydrogels for angiogenic GF release	[116-123]
Chitosan	Polysaccharide with tunable chemistry that allows for the control of degradation properties; low cost and easily available biopolymer with structural similarity to natural glycosaminoglycans; temperature/pH-sensitive gels can formed from quaternized chitosan and glycerophosphate, and used as an intelligent carrier	Chitosan forms hydrogels by physical cross-linking or chemical cross-linking which can incorporate GFs such as FGF; useful scaffold for injectable biological materials	[124-126]
	Synthetics		
PLGA	Good biocompatibility, biodegradability, low immunogenicity, low toxicity and mechanical strength; FDA-approved polymer for drug delivery	PLGA microparticles and solid scaffolds as controlled delivery platforms for VEGF-A, IGF-I, TGF-β1 and other GFs	[91, 127-132]
PEG-based synthetic biomaterial	Bioinert material explored as a non-degradable option in protein delivery; PEG can be readily conjugated with other natural and synthetic materials	PEG copolymers able to form environmentally sensitive hydrogels and to allow the attachment of biologically specific peptides to enhance control release of angiogenic GFs	[133-137]
Aminoacid-based polymers	Biodegradable materials that can be complexed with gelatin to prepare pH-sensitive matrices for controlled protein delivery	Poly(γ-glutamic acid)-sulfonate, gelatin-polylysine (gelatin-PLL) and gelatin-poly(glutamic acid) (gelatin-PLG) hydrogels for controlled delivery of FGF	[138, 139]
Polyacrylamide and derivatives	Thermosensitive polymers that undergo phase transition near the body temperature	Steric stabilization of liposomes; useful to deliver VEGF to human vascular ECs over an extended time period	[140, 141]

Table 4. Pre-clinical studies on targeted growth factor delivery systems for cardiac repair

Growth Factor	Delivery System	Animal model	Route	Effect	Reference
FGF-1	Peptide nanofibers	Acute MI in SD rats	IM	Treatment with FGF-1+p38 MAP kinase inhibitor: increased cardiomyocyte mitosis; reduced scarring and wall thinning with markedly improved cardiac function	[142]
	Slow release pump	Chronic MI in pigs	Perivascular space	Improved perfusion in the LCx region, but no significant blood flow in the LAD territory; no cardiac function and histology assessments	[143]
FGF-2	*p*(NIPAAm-co-PAA-co-BA) hydrogel	Acute MI in Fischer rats	IM	Improved angiogenesis and regional blood flow, but chronic inflammatory response observed near the polymer injection site	[141]
	Gelatin hydrogel	Chronic MI in Lewis rats	IM	Functionally significant angiogenesis and improved LV function	[144]
	Chitosan hydrogel	Acute MI in SD rats	IM	Recovered LVEF, enhanced arteriole density and significantly reduced infarct size and fibrotic area	[126]
	Chitosan hydrogel	Chronic MI in rabbits	Surface of the ischemic myocardium	Increased angiogenesis and evidence of enhanced collateral circulation in the ischemic myocardium	[125]
	Gelatin hydrogel	Acute MI in SD rats	IM	Improved vessel density; no differences in infarct size and fibrosis among the groups; no improvements on cardiac function	[145]
	Gelatin hydrogel microspheres	Chronic MI in pigs	IM	Positive LV remodeling and improved vascular density	[146]

VEGF-A	Anti-P-selectin-conjugated liposomes	Acute MI in SD rats	Tail-vein injections	Significant increase in tissue vascularization; no evidence of mature of angiogenic response	[147]
	Fusion Protein with a collagen-binding domain	Acute MI in SD rats	IM	Increased capillary density; no evidence of vasculogenesis	[148]
	p(PVL-b-PEG-b-PVL) hydrogel	Subacute MI in SD rats	IM	Attenuated adverse cardiac remodelling and improved ventricular function	[149]
	PLGA microparticles	Acute ischemia–reperfusion in SD rats	IM	Increased angiogenesis and arteriogenesis; a positive remodeling of the heart was also detected in the VEGF-A-microparticle group with a significantly greater LV wall thickness	[132]
	Core/shell nanoparticles	Subacute MI in SD rats	IM	Improved heart functions (ejection fraction and cardiac output)	[150]
VEGF-A/PDGF-BB	Alginate hydrogel	Subacute MI in Fisher rats	IM	Higher vessel density with sequential GF delivery than single factors; no increment in capillary density with sequential delivery of both proteins in alginate	[119]
EPO	cyclodextrin/MPEG–PCL–MPEG hydrogel	Acute MI in SD rats	IM	Reduced infarct size and improved cardiac function without evidence of polycythemia	[151]

FGF-1: acidic Fibroblast Growth Factor; FGF-2: basic Fibroblast Growth Factor; VEGF: Vascular Endothelial Growth Factor; PDGF: Platelet-derived Growth Factor; EPO: Erythropoietin; MI: myocardial infarction; SD: Sprague-Dawley; IM: Intramyocardial; LAD: Left anterior descending coronary artery; *p*(NIPAAm-co-PAA-co-BA): poly(N-isopropylacrylamide-co-propylacrylic acid-co-butyl acrylate); LVEF: left ventricle ejection fraction; *p*(PVL-b-PEG-b-PVL): poly (d-valerolactone)-block-poly (ethylene glycol)-block-poly (d-valerolactone); PLGA: poly(lactic-*co*-glycolic acid); MPEG–PCL–MPEG: [methoxy polyethylene glycol–poly(caprolactone)-(dodecanedioic acid)–poly(caprolactone)-methoxy polyethylene glycol] triblock polymer.

6.1.1) Hydrogels

Hydrogels are defined as three-dimensional polymer networks swollen by aqueous solvent, which is the major component of the gel system [152]. These systems may comprise an especially appealing class of delivery vehicle, as they can be introduced into the body with minimally invasive procedures and are often highly biocompatible, owing to their high water content [121, 153]. However, the localized and sustained release of GFs from conventional hydrogels is difficult because it depends on the cross-linking density and/or the degradation properties of the hydrogels. Consequently, initial burst release and deactivation of the released GFs are generally observed [120, 154]. Currently, research efforts are focused on the development of novel approaches that can control the release rate of GFs from carrier gels without changes in the physical and mechanical properties of the hydrogels.

Hydrogels of natural polymers have been used for delivering angiogenic cytokines. Collagen and its derivatives have commonly been used to deliver GFs by hydrogels. Gelatin is a denatured form of collagen that can be isolated from either bovine or porcine skin or bone by the partial hydrolysis of collagen [155]. Intramyocardial administration of FGF-2 loaded gelatine hydrogels induced functionally significant angiogenesis and improved left ventricular function in infarcted myocardium of rats [144] and pigs [156]. Gelatin hydrogels were also used to incorporate other GFs such as angiopoietin-1 [157] and erythropoietin (EPO) for cardiac repair. Regarding the latter, the application of gelatine hydrogel sheets containing EPO reversed left ventricular (LV) remodeling and improved LV function without inducing polycythemia in rat [158] and rabbit [159] chronic myocardial infarct models. These studies demonstrated that post-MI treatment with an EPO-gelatine hydrogel improves LV remodelling and

function by activating pro-survival signaling, anti-fibrosis, and angiogenesis without causing any side effect.

Fibrin is one of the major constituents of blood clots, which forms an immediate response to tissue injury, and therefore serves as a natural provisional platform for new cellular ingrowth. Because fibrin lyses slowly and locally, it has been used as a reservoir for GFs. In spite of some positive results with FGF or VEGF-A proteins in fibrin glue, the release kinetics of such preparations are indicative of an uncontrolled burst [122]. On the other hand, the addition of heparin to a fibrin gel has been useful for the sustained release and enhanced activity of angiogenic factors [160].

Angiogenic response was also detected when hyaluronic acid (HA) gels containing both VEGF-A and keratinocyte growth factor (KGF) were subcutaneously implanted into mice [114]. Regarding the myocardial injection of new biomaterials, a HA-based hydrogel was applied into the epicardium of the infarcted area of rats, resulting in a significantly decreased infarct size and apoptotic index [161]. In addition, HA hydrogels with tunable mechanics and gelation behavior have been investigated as a therapeutic material for cardiac repair in an ovine MI model [162].

Alginate-based hydrogels have been used as a localized delivery platform of angiogenic proteins. However, poor bio-resorbability has been reported as a disadvantage [105]. The VEGF bioavailability provided by an injectable alginate hydrogel led to a significant angiogenic response in ischemic hindlimbs [121]. Alginate hydrogels can also be tuned with other natural polymers such as chitosan and dextran becoming temperature/pH sensitive gels [163-165]. Such gels incorporating VEGF-A were stable and protein was released continuously, even after a month, without any initial burst release [120]. Injection of FGF-2 in a temperature-responsive chitosan

hydrogel was performed in rat [126] and rabbit [125] models of myocardial infarction resulting in positive cardiac repair.

Poly(ethylene glycol) (PEG), also known as poly(oxyethylene) or poly(ethylene oxide) (PEO), depending on its molecular weight, is one representative material which has been used to prepare synthetic polymer-based hydrogels loaded with angiogenic cytokines. Materials with Mw <100,000 are usually called PEGs, while higher molecular weight polymers are classified as PEOs. Several copolymers of PEG have been developed, such as 2-hydroxyethyl methacrylate, 1-vinyl-2-pyrrolidinone, and polyethylene glycol acrylate (HEMA–VP–PEG). This PEG-based hydrogel was examined as a matrix for the dual release of dexamethasone (DX) and VEGF. In this study, concurrent release of VEGF and DX was determined to be best from either VEGF/DX-loaded hydrogels or VEGF-loaded hydrogels with embedded PLGA microspheres containing DX [166]. In order to mimic the natural endogenous modulation in the release profile of angiogenic factors, heparin-conjugated polymers have been used in the formulation of hydrogels. Triblock copolymer of PEO and poly(propylene oxide) (PEO-b-PPO-b-PEO, commercially available as Pluronic or Poloxamer) has been used to incorporate FGF-2 into biodegradable Pluronic/heparin composite hydrogels, which induced proliferation of human umbilical vein endothelial cell (HUVEC) in addition to significant neovascularization when implanted into subcutaneous pockets in the dorsal side of Sprague-Dawley rats [167]. Moreover, Yamaguchi et al. reported the assembly, rheological properties, and targeted delivery/erosion profiles of non-covalently associated hydrogel networks produced via the interaction of a low-molecular weight heparin-modified star polymer (PEG-LMWH) and VEGF. The cytokine released from these hydrogels increased proliferation of

VEGF-responsive cell lines, suggesting a novel potential mechanism for targeted delivery and erosion via the release of therapeutically important protein cross-links in response to cell surface receptors [136]. In another strategy, VEGF was chemically coupled to PEG peptide hydrogel matrices to induce local angiogenesis by cross-linking matrix metalloproteinase (MMP) substrate peptides, providing retention of the factor in the matrix until its local release, triggered by active MMPs. Thus, the VEGF integrated to PEG peptide hydrogels could behave similarly to those in the natural ECM. When subcutaneously implanted in rats, these VEGF containing matrices, were remodeled into native and vascularised tissue [137]. In another elegant strategy, Wang et al. injected EPO into the rat infarcted myocardium using a supramolecular hydrogel self-assembled between alpha-cyclodextrin and metoxy polyethylene glycol-poly (caprolactone)-(dodecanedioic acid)-poly(caprolactone)-metoxy polyethylene glycol (MPEG-PCL-MPEG) triblock polymer (α-cyclodextrin/MPEG-PCL-MPEG). This hydrogel allowed a sustained release of EPO, which inhibited cell apoptosis and increased neovasculature formation, and subsequently reduced infarct size and improved cardiac function without evidence of polycythaemia [151]. Other synthetic materials used to prepare protein-loaded hydrogels are listed in Table 3.

6.1.2) Polymer scaffolds

Scaffolds are tridimensional matrices with a network architecture, useful to incorporate and release therapeutic proteins. Studies directed towards stimulating vascularization of implanted scaffolds have extensively explored polymeric matrices suitable for the sustained delivery of VEGF [131, 168-170]. GFs such as VEGF may be incorporated into scaffolds by two approaches. First, lyophilized VEGF is mixed with

polymer particles before processing the polymer into a porous scaffold, resulting in a rapid release (days to weeks in duration) of VEGF. The second approach involves pre-encapsulating the factor into polymer microspheres, and then fabricating scaffolds from these particles [131, 171-173]. The mechanism of VEGF incorporation into polymer scaffolds can determine the exposure duration and tissue distribution of the protein and, as a consequence, dictate the success of VEGF in therapeutic angiogenesis using scaffold platforms for its delivery. According to previous studies by Ennett et al., VEGF was positioned predominantly adjacent to scaffold pores when incorporated directly, and was rapidly released (40–60% in 5 days). After a small incision on the dorsal side of the rodent, scaffold was subcutaneously implanted into the pocket. On the other hand, pre-encapsulation led to the VEGF being more deeply embedded and resulted in a delayed release [131]. In fact, polymer scaffolds can act to confine microparticles at the defect site and can help maintain structural integrity during healing in addition to being biodegradable and biocompatible [108, 174].

Composite scaffolds, constituted by a synthetic biocompatible material, a poly(ether)urethane-polydimethylsiloxane blend, and a biological polymer, the fibrin, were also used to incorporate VEGF and FGF-2. The biological activity of the released GFs was maintained as demonstrated by HUVEC proliferation [175].

Many of the polymer delivery modules used to stimulate vessel ingrowth into scaffolds are able to deliver VEGF for periods greater than 2 weeks, but a disadvantage of these systems is the inability to determine whether VEGF release is complete. To date it has been unequivocally demonstrated that delivery of VEGF from a biopolymer, increases vessel ingrowth into porous scaffolds in a rat model of angiogenesis (implanted in the dorsal paramedian region of the skin), although the long-term stability

of the induced neovascularization within scaffolds after VEGF withdrawal has not been intensively investigated yet [169].

6.1.3) Nano- and microparticles

Reports have shown that GFs can promote localized angiogenesis *in vivo* if administered in a nano- or microparticulate depot [91, 116, 129, 176]. Polymeric nano- and microparticles are illustrated in Fig.4. These particulate delivery systems are considered potential tools to overcome the limitations of intravenous administration of therapeutic proteins. Poly(lactic-*co*-glycolic acid) (PLGA) copolymer is an attractive material to prepare cytokine-loaded particles because of its excellent biocompatibility and high safety profile [177-181]. Most GF delivery strategies using PLGA particles for angiogenesis have been performed in hindlimb ischemia models resulting in an increased blood vessel formation [182-184]. Also, the effect of delivery of PLGA microparticles loaded with VEGF-$A_{165,}$ has been studied in a rat model of cardiac reperfusion–ischemia. An increase in angiogenesis and arteriogenesis was observed in animals treated with VEGF microparticles, besides a positive remodeling of the heart with a significantly greater LV wall thickness [132]. PLGA has been also used to encapsulate heat shock protein 27 (HSP27), which has protective effects in cardiac cells under hypoxic conditions and in ischemia/reperfusion animal models [185]. HSP27 fused with transcriptional activator (TAT) was encapsulated into PLGA particles and the microsphere/alginate hydrogel combination delivery systems maintained protein bioactivity and recovered the proliferation of cardiomyoblasts cultured under hypoxic conditions [186]. A blend of PLGA and poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBV) was used to prepare HGF-loaded composite microspheres with a core-shell

structure. This system provided a sustained delivery of HGF with maintained bioactivity for at least 40 days [187]. Other GFs such as EPO [188] and FGF-2 [189] have been encapsulated into PLGA microparticles. In a different approach, VEGF was co-lyophilized with trehalose and rat serum albumin in succinate buffer to yield < 45 μm particles. These microparticles were incorporated into low molecular weight poly(trimethylene carbonate) and induced significant blood vessel formation when injected subcutaneously into the dorsal area of Wistar rats [190]. Recently, d'Angelo et al. have developed a new injectable controlled release device based on polymeric nanoparticles for the delivery of PDGF-BB and FGF-2. Incubation of these nanoparticles with EC culture models confirmed that these GFs were released in a bioactive form [191]. In another study, Tang et al. developed heparin-functionalized chitosan (CS)/poly(γ-glutamic acid) (γ-PGA) nanoparticles (HP-CS/γ-PGA nanoparticles) for multi-functional delivery of FGF-2 and heparin. Sustained release of FGF-2 from the nanoparticles enhanced the proliferation of human foreskin fibroblast cells (HFF) and angiogenic tube formation by HUVECs, suggesting the retaining of bFGF mitogenic activity [192]. Recently, a hyaluronic acid/chitosan polymer combination was also designed to prepare nanoparticles as delivery vehicles for VEGF and PDGF-BB, resulting in entrapment efficiencies of 94% and 54%, respectively [193].

PLGA microparticles have also been combined with other delivery systems in order to optimize the patterns of growth factor controlled release. Alginate gel/PLGA microsphere combination system containing VEGF enhanced the angiogenic response after hind limb ischemia in rats [194] and mice [195]. This combination system also allowed a dual delivery strategy and improved the effects of single factors. Also,

sequential release of VEGF and PDGF from alginate hydrogels led to a higher density of α-actin positive in a rat model of myocardial infarction but sequential administration of both free proteins did not achieve this response [119].

A microsphere/scaffold combination strategy has been tested using a porous PLGA scaffold capable of multiple GF delivery. In this approach, mixing particulate polymer and one factor with microspheres containing a pre-encapsulated second factor resulted in dual GF delivery with a distinct release rate for each factor [171, 196]. Recently, Saif et al. reported the development of injectable PLGA-based scaffolds releasing single factors or combinations of VEGF, HGF, and angiopoietin-1 with and without concomitant infusion of cord blood–derived vascular progenitors. Dual and triple combinations of scaffold-released GFs were superior to single release. Moreover, combined use of scaffold released GFs and cell therapy improved neovascularization in murine hindlimb ischemia models [197]. As other approach, gelatine microparticles incorporated within the porous network of a scaffold made of poly (propylene fumarate) has been evaluated as a delivery system for the controlled release of VEGF. Although marked burst release was observed, the relative amount of VEGF associated with gelatine achieved an equilibrium value with no strong dependence on its dose. These *in vivo* and *in vitro* release kinetics were characteristic of the specific GF due to the effects of VEGF size, charge, and conformation on its complexation with gelatine [108].

In an elegant strategy, Chung et al. developed a heparin-functionalized nanoparticle–fibrin gel complex containing VEGF, which increased angiographic score and collateral density in a rabbit model of hind limb ischemia [198].

6.2. Lipid-based growth factor delivery systems

Liposomes, solid lipid particles (SLN), and lipid nanocapsules (LNC) are different configurations of lipid-based nanoparticles, as illustrated in Fig.4. Despite the numerous approaches involving lipid-based formulations for protein delivery [199, 200], there are few reports dedicated to these systems as angiogenic proteins carriers for cardiac repair. On the other hand, the accumulation of liposomes in the areas of experimental myocardial infarction has been demonstrated [201-203]. Scott et al. developed anti-P-selectin-conjugated liposomes for targeted delivery of VEGF to the rat infarcted myocardium, resulting in significant increase in fractional shortening and improved systolic function [147]. In order to face the drawbacks of liposomes regarding clinical applications, particularly their instability and their interaction with high-density lipoproteins in blood, the design and characterization of polymer-supported liposomal systems have been described [150, 204, 205]. In this context, Oh et al. reported the formation of a temperature-induced gel composed of core/shell nanoparticles for regeneration of ischemic heart. The core was composed of lecithin containing VEGF and the shell was composed of Pluronic-127 (poly(ethylene oxide)-poly(propylene oxide)-poly(ethylene oxide) triblock copolymer. The inducement of the gel formation took place when Capryol 90 (propylene glycol monocaprylate) was added to an aqueous solution of the core/shell nanoparticles at body temperature. Although a minimum difference in neovascularization was observed between the core/shell nanoparticles and their gel, a comparable improvement in the recovery of heart function was observed with the gel system when applied to a myocardial infarction model in rats [150].

6.3. Other devices for growth factor delivery

Although the GF delivery systems described above are the most versatile and most intensively studied ones, a few other devices have also been utilized for GF delivery. For example, polymeric micelles, dendrimers and inorganic nanoparticles have been tested as delivery platforms. Micellar formulations have been used primarily for antitumor drug delivery in clinical or preclinical trials [206], but they are beginning to be explored for GF delivery. Lee et al. reported FGF-2 entrapment in heparin-conjugated Tetronic®- Poly(ε-caprolactone) polymeric micelles as an injectable vehicle for FGF-2 delivery [207]. Mesoporous silica nanoparticles (MSNs) have attracted attention for their unique structure features, including large surface areas, tunable pore sizes (2–10 nm in diameter), and well-defined surface properties [208]. In addition, MSNs have been approved by the FDA as a new biocompatible material. In a novel strategy, Zhang et al. developed an acid-modified water-in-oil microemulsion to encapsulate FGF-2 within MSNs *in situ*. As a result, high loading efficiency of FGF-2 into MSNs was achieved (around 70%) and the cytotoxicity test indicated that the MSNs are not toxic [209]. Recently, VEGF was conjugated to the surface of gold nanoparticles and this novel approach was studied in a murine ischemic hindlimb model. A 1.7-fold increase in blood perfusion besides increased capillary density was achieved after IV injection of VEGF-conjugated gold nanoparticles via the enhanced permeability and retention (EPR) effect [210]. A new externally-regulated delivery system was developed to explore sequential release of VEGF and sphingosine 1-phosphate (S1P), a GF that stimulates vascular stability. In this strategy, hollow cellulose acetate fibers promoted sequential delivery of factors and cellular recruitment and functional angiogenesis in a murine Matrigel plug model [211].

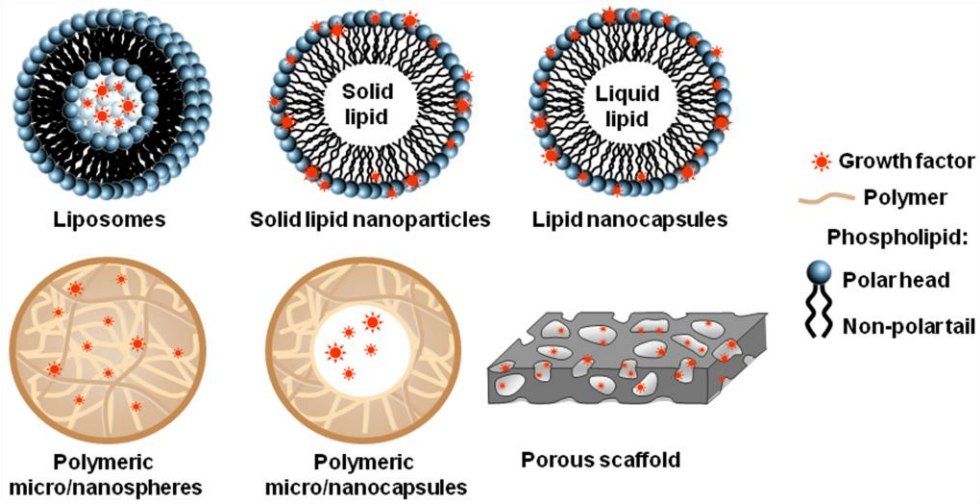

Fig. 4. Schematic representation of different drug delivery approaches used as platforms to deliver GFs.

6.4. Concluding remarks

The different growth-factor delivery systems listed above constitute an important result of intensive efforts to overcome limitations of protein-based therapy for therapeutic angiogenesis. The clearest drawback of GF therapy is the need to maintain bioactivity and therapeutic concentration to induce the desired effect within the required timing. Establishing the protein threshold concentration and its local exposure duration remains to be determined and represents the paramount challenge. The protein stability and pharmacokinetic issues may be solved or attenuated by incorporation of GF in natural or synthetic delivery matrices. However, on the basis of the pre-clinical studies, it is not yet possible to identify the better platform to deliver one or multiple GFs for cardiac repair. Some aspects such as material biocompatibility, protein stability and scale-up may be considered. However, substantial differences between animal models and humans further complicate the scenario. Over the past several years, many growth-

factor delivery strategies have been tested in pre-clinical studies. However, little information about clinical settings using protein delivery systems is available. Controlled release of FGF-2 encapsulated in heparin-alginate pellets led to significant angiogenesis with low systemic effects in patients undergoing bypass surgery, but this approach did not alleviate operative risks [212]. Therefore, further clinical trials to evaluate the effects of treatment induced by controlled GF delivery methods may be necessary.

Even though the pathway to reach optimum protein therapy is not free of hindrances, intensive research in rational protein design technology and new biopolymers and nanomaterials for controlled release of proteins will enable significant progress in the efficacy and safety of known and new GFs applied to cardiac repair. Therefore, treatment of IHD with a single protein or, most likely, with a combination of multiple proteins incorporated into delivery systems may become an effective therapy in the future.

7. FUTURE DIRECTIONS

More than a decade has passed since the first clinical trial employing an angiogenic treatment for IHD was carried out. Since the first clinical studies many questions have emerged. First of all, researchers agree on the need to explore in detail the mechanisms involved in the complex process of angiogenesis. Refined techniques now being perfected such as microarray analysis, proteome and secretome profiling, as well as cell sorting and image analysis will play a major role in achieving this goal [213]. The improvement in our knowledge of the angiogenesis pathways will allow us to find new and better targets. But as our knowledge grows, the difficulty of integrating all the

notions involved becomes more evident. To overcome this handicap, which is a result of the interrelation between different factors and pathways, it has been proposed that we should combine quantitative biological experiments and computational models. This systems biology approach can also deal with the individual variability inherent in the ischemic disease population [213].

Angiogenesis is a complex, multi-step process, and various factors are critical at each stage, which indicates that a more effective therapeutic angiogenesis could be achieved by employing multiple growth factor delivery. Once the target has been defined, the issue of what the best therapeutic approach is still remains unclear. However, in the literature there is a certain agreement on the promising role of protein therapy, combined with drug delivery systems. Nevertheless, several questions remain which require solutions before we can move from the bench to the bedside. As a critical starting point, producing and purifying proteins in a large scale manner is a difficult task, particularly as regards the requirements for clinical use, and the economic cost of these processes. The use of bioreactors or high throughput column isolation offers a possible solution [213].

To take a step forward, some authors propose combining both cellular therapies and protein delivery systems. In this case it is important to establish well defined protocols for obtaining and culturing the cells, as well as studying the optimal number of cells to be administered and avoiding incompatibility concerns [214]. In any case, from the clinical trials conducted until now one of the main conclusions has been that it is essential to find the suitable moment to treat the patient, considering the physiological process that follows IHD, and how it might affect the treatment.

Another important issue is the question of evaluating the progression of angiogenic therapy in the heart. A fast growing field in angiogenesis assessment is imaging technology (reviewed in [215]). The improvements accomplished in terms of sensitivity and specificity will result in a better understanding and explanation for the findings of the clinical trials, which are often contradictory. Cardiac magnetic resonance appears to be a very suitable test to assess spatial and temporal changes after angiogenesis therapy. Also, when combined with complementary techniques, this method can provide essential information about physiology, morphology and metabolism [216].

A great effort to obtain beneficial effects in patients as a result of therapeutic angiogenesis has been made over the last 30 years. The number of research areas working in unison to achieve this goal increases as the knowledge does so, and hopefully it will continue this way, until we find the cure to ischemic heart disease. In this context, and despite all the handicaps mentioned above, in our opinion, the drug delivery systems employed to administer and control protein release appear to offer a promising strategy. For cardiac repair purposes, where revascularization and myocardium regeneration are lasting complex process, the encapsulation of GFs into polymeric microparticles shows crucial advantages. As it offers cytokine protection against physical, chemical or enzymatic degradation, it is possible to maintain therapeutic levels over a longer period of time, thus minimizing the dosage and reducing potential adverse effects. Gene or cell therapy shows some limitations including the lack of control over the dose. In contrast, polymeric microparticles make it possible to control the amount of protein administered. Moreover, it is possible to predict the levels of released protein and to alter them by modifications in the raw materials and structure of the particle, to mimic the proper environment in which the

tissue can be regenerated. Another important fact is the need for multiple GFs to complete the angiogenic process. It is also possible to combine different GFs loaded into polymeric microparticles with different properties, achieving the most suitable release profile for each GF. In addition, the particle size can be fitted to cardiac administration and modifications in the microparticle surface (such as pegylation) can avoid macrophage uptake of microparticles in the injured heart tissue surrounding. Regarding the cost-effectiveness of the industrial production, various advantages can also be listed: high availability of polymers (including low cost ones), feasible preparation methods, possibility of scaling-up the process, etc. In conclusion, polymeric microparticles seem to be adequate to fulfil most of the requirements that the ideal delivery system must have (reviewed in [217]).

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HYPOTHESIS AND OBJECTIVES

The clinical trials performed in patients with myocardial infarction and based on the intravascular injection of growth factors have failed, owing, among other reasons, to protein instability after injection. The hypothesis of this research is that a local controlled release of the growth factors by using a polymeric delivery system could protect the growth factors from degradation and stimulate cardiac repair. To test this, the following specific objectives were proposed:

1. Design, development and physico-chemical characterization of PLGA microparticles intended for intramyocardial administration. *In vivo* compatibility assessment of the developed microparticles with the cardiac tissue.

2. Development of $VEGF_{165}$ loaded PLGA microparticles, *in vitro* characterization and assessment of the potential benefit of the $VEGF_{165}$-microparticles in an acute rat myocardial ischemia–reperfusion model.

3. Development of FGF-1 and NRG-1 into PLGA microparticles, *in vitro* characterization and evaluation of the therapeutic potential of FGF-1 and/or NRG-1 cytokines delivered from PLGA microparticles in a rat myocardial infarction model.

CHAPTER 1

CHAPTER 1

PLGA microparticles as cardiac delivery systems: preparation, characterization and *in vivo* assessment

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ABSTRACT

Growth factor-based therapy is a promising strategy to treat patients with myocardial infarction. The development of growth factor delivery systems remains an important challenge in the field of biomaterials for cardiac regeneration. In this work, we developed poly-lactide-*co*-glycolide (PLGA) microparticles (MP) by Total Recirculation One-Machine System (TROMS) after the preparation of a multiple emulsion. TROMS produced PLGA-MP in the size range of 2-30 µm varying apparatus conditions. Particles were negatively charged (–30 mV at pH 7.6) with minimal content of residual poly(vinyl alcohol) (PVA). Next, we assessed the potential of these particles for its administration in the heart, in a rat model of myocardial infarction. Microparticles with a diameter of 5 µm were determined as the most compatible for intramyocardial administration in terms of injectability and tissue response. They were also present in the heart tissue for up to one month post-implantation. CD68 immunolabeling revealed 31% and 47% microparticle uptake one week and one month after injection, respectively ($P<0.001$). Taken together, these findings support the feasibility of PLGA-MP as potential vehicles for cardiac drug delivery, paving the way for delivering growth factors in the myocardium.

Key words: Myocardial infarction, PLGA microparticles, biocompatibility, phagocytic uptake, growth factors.

1. Introduction

Myocardial infarction (MI) is a great threat to life in the developed countries, and so research efforts are being focused on the development of new therapies. Therapeutic angiogenesis induced by exogenous administration of growth factors such as vascular endothelial growth factor (VEGF) and fibroblast growth factor (FGF) has been considered a promising strategy to treat patients with MI. However, although many pre-clinical studies have reported beneficial effects of angiogenic growth factor administration after MI, neither VEGF nor FGF have shown efficacy in double-blinded clinical trials [1, 2]. These disappointing results were attributed, at least partially, to the high intrinsic instability of the protein when systemically administered and the short half-life during which growth factors retain their biologic activity *in vivo*.

Current methods for growth factor delivery require administration of high protein concentration and repeated injections which may result in abnormal vessel formation and unwanted side effects such as hypotension [3, 4]. Therefore, targeted delivery of angiogenic proteins into the ischemic heart could be useful. Delivery strategies that provide a sustained local release of growth factors would not only control protein concentration, but could also minimize systemic exposure. A number of approaches have been designed to deliver growth factors in the heart in a controlled fashion. These include hydrogels, peptide nanofibers, liposomes, nano- and microparticles mainly for delivery of VEGF [5-9], FGF-1[10] and FGF-2 [11, 12]. While each delivery platform has both merits and drawbacks in the controlled delivery of angiogenic growth factors, there are few reports about the feasibility of these approaches via the intramyocardial route in relation to injectability, local retention and tissue response.

Polymeric microparticles encapsulating protein drugs offer the possibility of controlling the release of delicate macromolecules over extended time periods [13]. Copolymers of lactic and glycolic acids (PLGAs) have been studied most commonly for this purpose because of their proven safety record and established use in marketed products for controlled delivery of several peptide drugs [14, 15]. Nevertheless, PLGA microparticles (MP) have not been thoroughly investigated as a feasible delivery system for growth factors into the myocardium.

In this study, we evaluate the compatibility of PLGA-MP with intramyocardial administration and the macrophage-mediated phagocytosis of these particles using a rat model of myocardial infarction. To this end, we developed PLGA-MP by Total Recirculation One-Machine System (TROMS), a technique based on the multiple emulsion solvent evaporation method and suitable for the encapsulation of labile molecules like proteins [7, 16].

2. Materials and Methods

2.1 Materials

PLGA with a monomer ratio (lactic acid/ glycolic acid) of 50:50 Resomer® RG 503H (M_w: 34 kDa) was provided by Boehringer-Ingelheim (Ingelheim, Germany). Polyethylene glycol (PEG; M_w: 400), human serum albumin (HSA) and rhodamine B isothiocyanate were provided by Sigma-Aldrich (Barcelona, Spain). Dichloromethane and acetone were obtained from Panreac Quimica S.A. (Barcelona, Spain). Poly(vinyl alcohol) (PVA) 88% hydrolyzed (M_w: 125,000) was obtained from Polysciences, Inc. (Warington, USA). Dubelcco's Modified Eagle Medium (DMEM) was from Gibco-

Invitrogen (Carlsbad, CA, USA). Mouse monoclonal anti-rat CD68 antibody (MCA341R) was purchased from Serotec (Oxford, UK). Alexa Fluor 488 goat anti–mouse IgG antibody was provided by from Molecular Probes (Eugene, OR, USA).

2.2 Microparticle formulation

Microparticles were obtained after the preparation of a multiple emulsion ($W_1/O/W_2$). The inner aqueous phase (W_1) consisted of 5 mg of HSA and 5 µl of PEG 400 dissolved in 200 µl of phosphate-buffered saline (PBS). The organic phase was composed of 50 mg of PLGA dissolved in 2 ml of a dichloromethane/acetone mixture. The outer aqueous phase (W_2) was 20 ml of a 0.5%$_{w/v}$ PVA solution. For fluorescence-labeled microparticle formulation, rhodamine B isothiocyanate (0.5 mg/ml) was added to W_1 phase.

2.3 Microparticle preparation

Non-loaded PLGA microparticles were prepared using the Total Recirculation One-Machine System (TROMS) [7, 16, 17]. Briefly, the organic phase was injected into the W_1 phase by means of a glass needle with a Teflon plunger. Next, the previously formed inner emulsion (W_1/O) was recirculated through the system under a turbulent regime maintained by a pumping flow though a needle with an inner diameter of 0.17 mm. After this homogenization step, W_1/O emulsion was injected into the W_2 phase. The turbulent injection though a second needle resulted in the formation of a multiple emulsion ($W_1/O/W_2$), which was allowed to circulate through the system to become homogeneous. The multiple emulsion was stirred for 3 h to allow solvent evaporation. Microparticles were washed three times with ultrapure water by consecutive

centrifugation at 4 °C (20,000×g, 10 min). Finally, the particles were resuspended in 1 ml of ultrapure water, frozen at −80 °C, lyophilized (Genesis 12EL, Virtis) and stored at 4 °C. In order to obtain batches with different particle sizes, we adjusted the following TROMS parameters during microparticle preparation: pumping flow, recirculation times to form the both W_1/O and $W_1/O/W_2$ emulsions, and the inner diameter of the needle to prepare the $W_1/O/W_2$ emulsion. A needle with inner diameter of 0.17 mm was used to form the primary W_1/O emulsion of all microparticle batches.

2.4 Size, surface charge and imaging

Particle size and size distribution of the microparticles prior to lyophilization were measured by laser diffractometry using a Mastersizer® (Malvern Instruments, UK). When the particle size was measured after lyophilization, the particles were resuspended in deionized water before measurement. The average particle size was expressed as the volume mean diameter in micrometers and samples were measured in triplicate.

For rhodamine-labeled microparticles, particle size was also estimated using the software imaging system Cell* connected to the camera fluorescence microscopy system CH40 (Olympus GmbH, Münster, Germany).

Particle surface charge was determined by zeta potential measurement (Zeta Plus Potential Analyzer, Brookhaven Instruments Corp., New York, USA). A dilute suspension (0.5 mg/ml) of microparticles was prepared in 1 mM KCl (pH = 7.6) and the zeta potential measurements were performed after 10 cycles in the high precision mode.

Rhodamine-labeled microparticles were imaged at high-power by fluorescence microscopy. A random sample of microparticles was resuspended in water, mounted on a microscope slide, and visualized using a camera microscopy system (Olympus CH40).

2.5 Residual PVA Content

The residual PVA associated with microparticles was determined by a colorimetric method [18]. Two milligrams of dry microparticles were hydrolyzed with 2 ml of 0.5 M NaOH for 15 min at 60ºC. The solution was then neutralized with 900 µl of 1 N HCl and the volume was adjusted to 5 ml with distilled water. Next, 3 ml of a 0.65 M solution of boric acid, 0.5 ml of a solution of I_2/KI (0.05 M/0.15 M) and 1.5 ml of distilled water were added. These conditions allowed the formation of a colored complex between two adjacent hydroxyl groups of PVA and an iodine molecule. After 15 min of incubation, the absorbance was measured at 690 nm using an Agilent 8453 UV–visible spectrophotometer (Agilent technologies, Palo Alto, CA, USA). A standard plot of PVA was prepared under identical conditions and measurements were performed in triplicate.

2.6 In vivo studies using non-loaded PLGA-MP

2.6.1 Determination of microparticle injectability

Prior to *in vivo* studies, microparticle dispersibility was tested in three different resuspension media: PBS, DMEM and DMEM supplemented with a surfactant mixture composed of 0.1%$_{w/v}$ carboxymethylcellulose, 0.8% $_{w/v}$, polysorbate 80 and 0.8% $_{w/v}$ mannitol in PBS, pH 7.4 (DMEM-S). Microparticle suspension injectability was assessed by its ability to pass through a 29-gauge needle [19]. Particle concentration and

particle size were evaluated as injectability parameters in order to define the optimal microparticle formulation for heart injection.

2.6.2. Myocardial infarction model

Animal experiments were carried out using a rat model of cardiac acute-reperfusion–ischemia. Rats were initially anesthetized with 4% isoflurane in an induction chamber. Prior to surgery, animals received analgesic drug ketoprofen 5 mg/Kg subcutaneously, fentanyl 0.15 mg/kg and heparin 0.1 mg/kg both administered by intraperitoneal route. The rats were then intubated and ventilated at 90 cycles/min (1.5–2% isoflurane was maintained for continuous anesthesia). A left thoracotomy through the fourth intercostal space was performed, and the left anterior descending (LAD) coronary artery was occluded 2–3 mm distal from its origin for 1 h and then re-opened. The chest was then closed in layers and rats allowed to recover on a heating pad. All animal procedures were approved by the University of Navarra Institutional Committee on Care and Use of Laboratory Animals as well as the European Community Council Directive Ref. 86/609/EEC.

2.6.3 Intramyocardial administration of microparticles

Six hours after LAD coronary artery occlusion, animals were assigned to receive microparticles of different sizes (30, 14, 5 and 2 μm) or medium alone. Microparticle suspensions (2 mg/100 μl) were injected with a 29-gauge needle into 4 regions in the border zone surrounding the infarct while the heart was beating. Prior to injection, freeze-dried microparticles were dispersed in the medium previously selected in the

injectability studies (DMEM-S). Three animals were included in each group. The chest was closed and rats were allowed to recover on a heating pad.

2.6.4 Histological assessment

Four days post-injection, animals were sacrificed and their hearts were collected for histology. After being harvested, the hearts were perfused-fixed in 4% paraformaldehyde at 4 °C, and sliced in three 4-mm-thick segments from apex to base. The hearts were dehydrated in ethanol 70% at 4 °C, embedded in paraffin and cut 5-µm-sections. Hematoxylin–eosin (HE) staining was carried out to localize the microparticles and to visualize tissue structure. Samples from control zone, right ventricule, and other organs (kidney, liver and spleen) were also analyzed.

2.6.5 In vivo phagocytic uptake of microparticles

A group of infarcted animals was injected with 5 µm-sized fluorescent-labeled microparticles and sacrificed 8 and 30 days later. Rhodamine B was used as a fluorescent marker to localize the injected microparticles by confocal microscopy in the heart tissue. After the hearts were frozen in OTC compound, frozen sections were prepared. In order to assess the phagocytic uptake of microparticles after their intramyocardial delivery, rat macrophage staining was carried out. Immunolabeling was performed with mouse anti-rat CD68 primary antibody (diluted 1:100 in TBS). Next, fluorescent Alexa Fluor 488 goat anti–mouse IgG secondary antibody was applied to sections followed by nucleus staining with TOPRO-3 (diluted 1:50 in PBS-glycerol). For confocal microscopy, a LSM 510 META (Carl Zeiss, Minneapolis, USA) microscope was utilized. Phagocytic uptake of the injected microparticles was

expressed in terms of microparticle phagocytosis index determined as the ratio of rhodamine-loaded microparticles internalized into CD68-positive macrophages to the total number of microparticles counted in each section. Eight serial sections of each rat were analyzed.

3. Results and discussion

3.1 Non-loaded PLGA-MP prepared by TROMS

A wide range of formulation methods have been used for encapsulating proteins into PLGA-MP. These include solvent extraction, phase separation, spray drying, solid encapsulation, static mixer extrusion, and expansion in a supercritical fluid. But the most frequently utilized method for the entrapment of fragile molecules is the water/oil/water (W/O/W) multiple emulsion solvent evaporation method [20]. Based on this method, TROMS has the advantage, over the conventional solvent evaporation techniques, of encapsulating compounds without the need for aggressive techniques or heating during the emulsification process. Thus, the method is especially useful for the encapsulation of fragile molecules such as growth factors. Previously, we successfully encapsulated VEGF and GDNF into PLGA-MP using TROMS, which maintained their biological activities [7, 16, 21].

In this study, TROMS produced microparticles in the size range of 2-30 μm varying apparatus conditions during microparticle production. Particle size remained unchanged after lyophilisation. The needle diameter for $W_1/O/W_2$ emulsion formation, pumping flow and recirculation times of both W_1/O and $W_1/O/W_2$ emulsions yielded batches with different particle sizes (Table 1).

Table 1. Influence of TROMS conditions on the final particle size

Batch #	Pumping flow (ml/min)	Needle diameter* (mm)	Recirculation times (min)		Mean size (μm)
			W_1/O	$W_1/O/W_2$	
1	25	0.50	3	4	30.1 ± 2.4
2	25	0.25	3	4	20.4 ± 1.8
3	25	0.12	3	4	21.4 ± 1.4
4	30	0.17	3	6	4.1 ± 0.7
5	30	0.17	2	6	14.7 ± 1.6
6	30	0.17	2	4	19.8 ± 2.6
7	30	0.17	3	4	5.1 ± 1.4
8	35	0.17	3	4	3.3 ± 0.9
9	50	0.17	3	4	2.0 ± 0.8

*Corresponding to the conditions for $W_1/O/W_2$ emulsion formation. A needle with diameter of 0.17 mm was employed in W1/O emulsion formation for all batches.

The inner diameter of the needles is a critical factor determining the final size of microparticles prepared by TROMS [17]. Microparticles with a diameter around 30 μm (batch 1) were obtained using the largest needle diameter to form the multiple emulsion (0.50 mm). A slight reduction in particle size was observed with needle diameters of 0.25 mm and 0.12 mm (batches 2 and 3, respectively, compared with batch 1). Table 1 also shows the influence of recirculation times of both W_1/O and $W_1/O/W_2$ emulsions on the final size of microparticles, which was strongly dependent on the recirculation time of the primary W_1/O emulsion. A reduction of 1 min on the recirculation time of this emulsion increased the particle size from 4.1 μm (batch 4) to 14.7 μm (batch 5). The pumping flow also played a key role in the final size of the microparticles, whereas increasing flows led to more turbulent regimes to form both primary and multiple emulsions. Consequently, smaller microparticles were formed under higher homogenization energies supplied by more vigorous flows.

Colloidal stability was analyzed by measuring the zeta potential of PLGA microparticle's surface. Particles were negatively charged (around –30 mV at pH 7.6)

and no significant differences in zeta potential values were observed among all TROMS-produced microparticles batches.

The morphology of the microparticles was examined by fluorescence microscopy (Fig. 1). Microparticles appeared spherical in shape and red fluorescence was distributed in the polymer matrix, indicating the maintenance of the fluorescent signal after rhodamine encapsulation.

Concerning the residual PVA content, the percentage of PVA recovered in the microparticles ranged from 1.1% to 1.6% depending on the formulations (results not shown). These values are several times lower than $13\%_{w/w}$ PVA content previously reported [22].

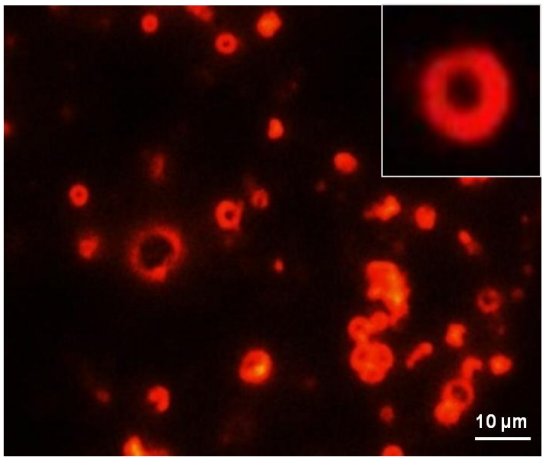

Fig. 1. Image of microparticles labeled with rhodamine under fluorescent microscopy

3.2 Injectability of TROMS-produced microparticles

Physical stability assessment was performed in order to select an adequate injectable PLGA microparticle suspension for local myocardial injection. Dispersibility of freeze-dried microparticles was tested in PBS, DMEM and DMEM-S. Microparticles were better dispersed in DMEM-S, probably due to the surfactant mixture added to

medium, which increased the viscosity of DMEM and reduced the particle sedimentation. Thus, DMEM supplemented with carboxymethylcellulose, polysorbate 80 and mannitol was selected as injection medium for animal experiments. Dispersibility and injectability of microparticles in DMEM-S were evaluated as function of particle concentration and particle size. Regarding the former, microparticle suspension in the range of 2−5 mg/100 µl was found to be both dispersible and injectable. Particle batches of 14 µm and 30 µm exhibited some resistance to resuspension in DMEM-S compared to smaller ones, probably due to sedimentation between particles >14 µm. Concerning the ability of microparticles to pass through a 29-gauge needle, moderate levels of sedimentation could also explain the resistance observed when 30 µm-sized particles passed through the needle. In contrast, microparticles of 2 µm and 5 µm were flowable. They can be easily injected through a 29-gauge needle, a typical needle size for heart injection [11].

3.3 Histological evaluation of injected microparticles into the myocardium

Microparticle batches of 30, 14, 5 and 2 µm were tested according to their compatibility with an intramyocardial injection. As mentioned above, large microparticles did not exhibit suitable resuspension in the injection medium. Indeed, blockages in the 29-gauge needle were detected during the administration of 30 µm-sized particles in the myocardium. Probably, the presence of aggregates obstructed the flow through the needle, which limited the injection of large microparticles in the rat heart. Despite the obstruction, a residual amount of 30 µm-sized particles reached the infarcted area, but these particles caused extensive damage to the surrounding myocardial tissue. As it can be seen in Fig. 2, HE staining showed a more consistent

accumulation of inflammation-mediated cells (IMCs) after injection of large microparticles (30 μm) compared to smaller ones (2 μm). In fact, large microparticles caused extensive myocardial necrosis in a porcine model [23]. On the other hand, there were fewer IMCs in the surrounding areas of 2 μm-sized particles, which did not induce severe responses. However, these particles with a diameter of 2 μm exhibited a low persistence in the heart, probably due to local phagocytic activity. Taken together, these observations demonstrated that particles with an intermediate diameter could be adequate for heart injection. Consequently, we selected 5 μm-sized particles to be injected into infarcted myocardium. Interestingly, they did not induce inflammatory reactions when compared to injection medium alone. Fig. 3 shows slightly inflamed areas provoked by the needle during the myocardial administration of DMEM-S alone or suspension of microparticles with a diameter of 5 μm. This finding correlates with the reported approach that 7 μm resin particles encapsulating FGF-2 did not cause myocardial damage [24]. However, the clinical application of these microparticles is limited because the resin material is non-biodegradable, unlike PLGA which degrades generating monomeric acids (lactic and glycolic acids) that are consequently eliminated from the body as carbon dioxide and water [25]. Importantly, no myocardial hemorrhage was observed in our HE sections. It is also worth noting that the injected 5 μm-sized particles did not injure other organs such as kidney, liver and spleen. Additionally, signs of physiological disturbances such as fibrillation upon microparticles injection were not observed.

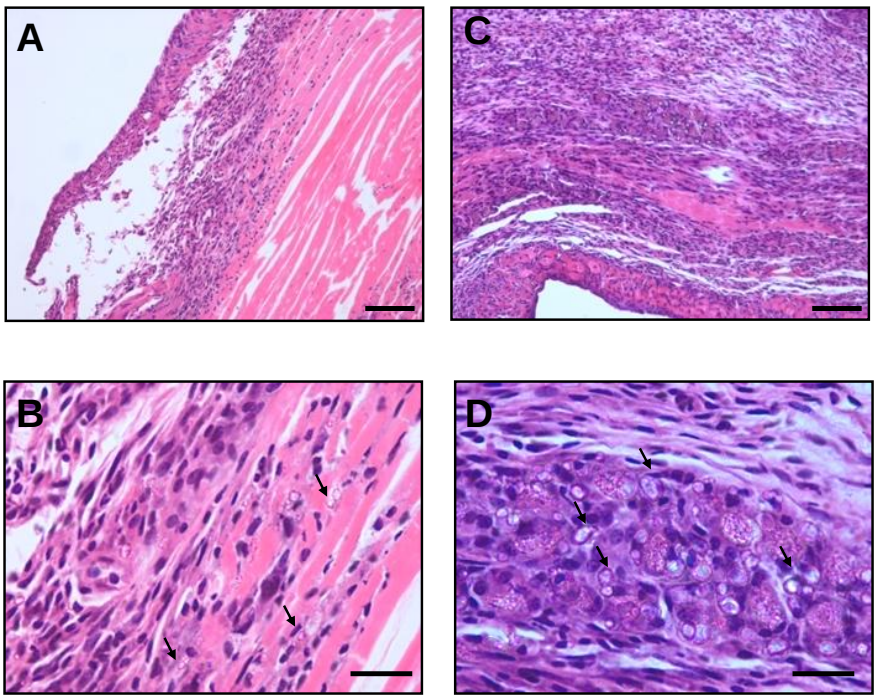

Fig. 2. Histological evaluation of excised heart tissue samples from the site of injection 4 days after microparticle administration. Microparticles with a diameter of 2 µm (A, B) and 30 µm (C, D) are clearly visualized at high magnification (B, D indicated by arrows). Scale bars: 20 µm (A, C) and 100 µm (B, D).

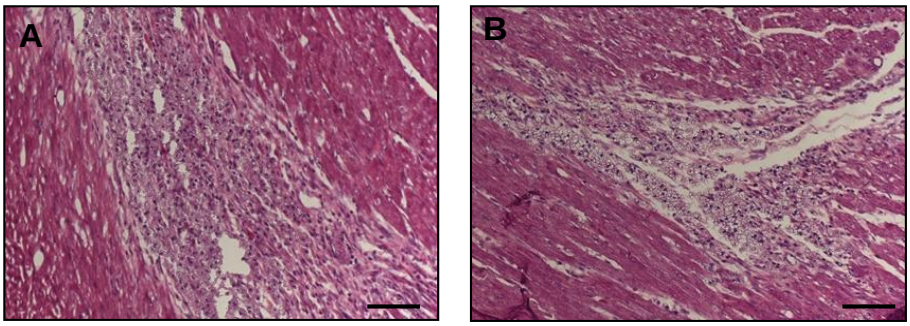

Fig. 3. Inflammatory reactions provoked by the injection medium alone (A) and microparticle suspension (B) in the rat myocardium visualized in HE sections. No differences in the accumulation of inflammatory cells were observed between areas surrounding the injection medium and injected microparticles. Scale bars: 50 µm.

3.4 Tissue retention and phagocytic uptake of microparticles

A representative image of an acutely ischemic rat heart injected with microparticles is shown in Fig. 4. Immediately after microparticle administration, a persistent blanching surrounding the injection point was observed. This indicates the change of color from dark pink to light pink of the tissue after polymer injection. No microparticle loss or leakage was observed, indicating a localized retention of microparticle suspension in the epicardial zone. Thereafter, confocal microscopy was performed to evaluate the temporal retention of PLGA-MP in the heart tissue. The fluorescent-labeled microparticles were visible for up to a month post-implantation (Fig.5). This is a significant temporal retention, whereas Sy et al. reported retention of poly(cyclohexane-1,4diyl acetone dimethylene ketal) (PCADK) microparticles for up 10 days in the myocardium [26]. Thus, our tissue retention results indicate the capacity of the PLGA-MP to remain in the myocardium for a prolonged period of time, a requirement for sustained growth factor treatment. Correlating with histological observations of HE sections, no fluorescent signal of rhodamine-loaded microparticles was observed in other tissues such as kidney, liver and spleen indicating no migration of the microparticles towards solid organs. This is an important feature of PLGA-MP for local delivery of therapeutics into myocardium, preventing systemic side effects of growth factors.

While there are reports that have described the phagocytic uptake of PLGA-MP in macrophage cultures [27, 28], there is no detailed *in vivo* study on the macrophage-mediated phagocytosis of PLGA microparticles in the heart tissue. As the macrophage is a primary responder cell involved in the regulation post-MI wound healing, eliminating apoptotic/necrotic myocytes and other debris [29], phagocytic activity of

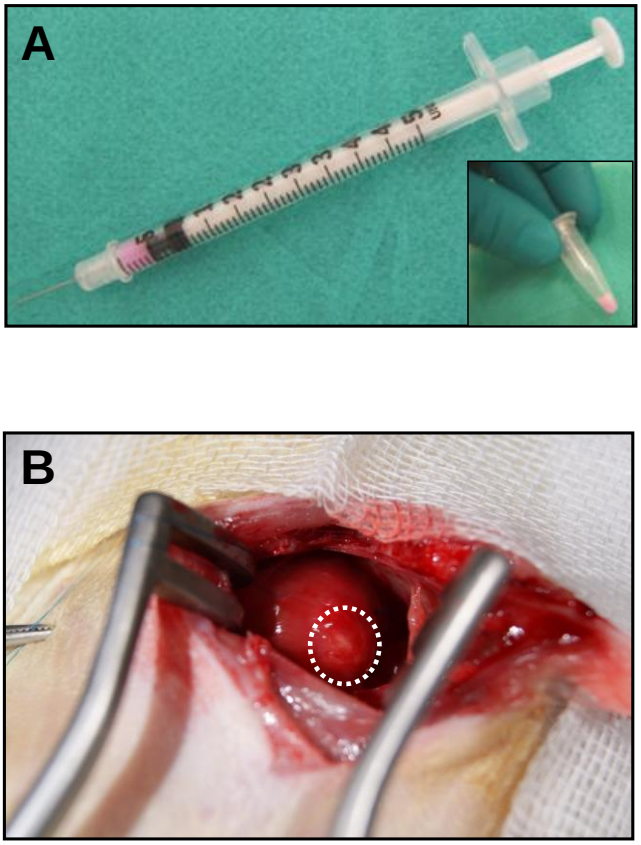

Fig. 4. Representative image of an acutely ischemic rat heart injected with non-loaded PLGA microparticles. Microparticle suspension prepared immediately before heart injection using a 29-gauge needle syringe (A). Note the extent of blanched tissue surrounding the injection point (dotted circle), indicating the tissue retention of polymer in the epicardial zone (B).

cardiac macrophages upon injected microparticles was further assessed. Quantification of phagocytic uptake of rhodamine-loaded microparticles was carried out by detection of CD68 macrophages. The extent of phagocytosis was assessed in two groups of animals: rats sacrificed one week or one month after intramyocardial administration of fluorescent microparticles. CD68 immunolabeling revealed a microparticle uptake of around 31% one week after microparticle injection (Fig. 5A,C). An increase in the phagocytic activity of macrophages upon microparticles was detected one month after

its administration, with a microparticle phagocytosis index around 47% (Fig. 5B-C, $P<0.001$).

In rodent models of myocardial infarction, within the first hours to 1 day, there are robust up-regulations of intramyocardial cytokines including tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), and interleukin-6 (IL-6). These cytokines mediate the acute remodeling process in the infarcted myocardium, which includes modulation of cardiac macrophages and phagocytosis [30]. After the initial increase of its gene expression in the infarcted region, the cytokines normally begin to decrease toward baseline after 1 week [31]. Therefore, a decrease in the phagocytic uptake of microparticles 1 month after injection must be expected, because of decreased phagocytic activity of cardiac macrophages during the chronic remodeling post-MI. However, a higher number of microparticles internalized into CD68-positive macrophage was quantified in injected animals after 1 month compared with 1 week. One potential explanation is that microparticles maintained their spherical shape and diameter around 5 µm, presenting some resistance to phagocytosis one week after injection, a very short period for polymer degradation. In contrast, one month after their injection, particles originally 5 µm in size were observed as smaller ones (< 2 µm) due to higher polymer degradation. Consequently, these small particles in the heart tissue were more susceptible to phagocytosis, whereas particle size around 1 µm is suitable for efficient uptake by macrophages [32].

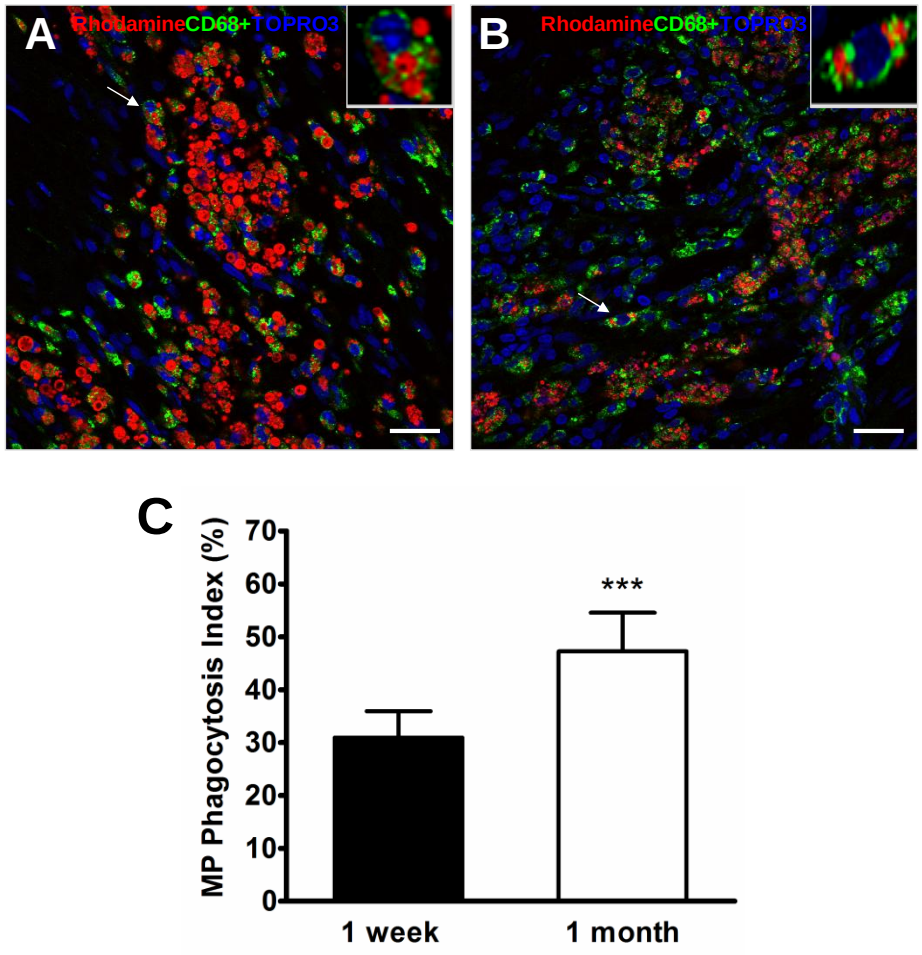

Fig. 5. *In vivo* phagocytic uptake of microparticles**.** Representative pictures of CD68 (green) macrophage immunofluorescence of heart sections 1 week (A) and 1 month (B) after intramyocardial administration of microparticles labeled with rhodamine (red). Note the fluorescent microparticles internalized into CD68-positive macrophage (inserts). Nuclear staining was performed with TOPRO-3 (blue). Scale bars: 20 µm. Quantification of microparticle (MP) phagocytosis was determined as the ratio of rhodamine-loaded microparticles internalized into CD68-positive macrophage to the total microparticles counted in each section (C). ***$P<0.001$.

On the other hand, in addition to particle size, other formulation parameters could affect the phagocytic uptake of microparticles. For example, particle hydrophobicity decreases with the amount of residual PVA associated with microparticles, reducing their recognition by macrophages [18]. We used a 0.5% PVA solution as stabilizer, which prevents microparticle coagulation during solvent removal. Using this low PVA concentration, we obtained microparticles with minimal content of

residual PVA. Moreover, microparticles presented a high negative charge, which is associated with a stable colloid nature. An increase in PVA concentration used for microparticle formulation would result in the increase in the residual PVA content. However, as PVA is a potentially toxic non-biodegradable polymer, its administration should be minimized as much as possible [33]. Therefore, changes in formulation parameters must be rationally performed to alter microparticle hydrophobiticy, aiming to control its phagocytic uptake.

4. Conclusions

In this study, we developed a PLGA microparticle formulation compatible with an intramyocardial injection in terms of particle size, injectability and tissue response. In addition, these particles exhibited the capacity to remain in the myocardium for up to one month. Concerning *in vivo* phagocytic uptake of microparticles, a moderate level of macrophage-mediated phagocytosis of PLGA microparticles was observed in the heart tissue. In particular, this result helps us to understand better the heart tissue response to a polymeric delivery system in the context of biomaterial research for cardiac regeneration. Thus, although PLGA microparticles designed in this study have offered significant potential as cardiac drug delivery systems, they can still be optimized to minimize its macrophage clearance. With this in mind, our further studies are now in progress aiming to develop particles with a more hydrophilic surface, by using PEGylation strategy.

Acknowledgments

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CHAPTER 2

CHAPTER 2

Sustained release of VEGF through PLGA microparticles improves vasculogenesis and tissue remodeling in an acute myocardial ischemia-reperfusion model

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ABSTRACT

The use of pro-angiogenic growth factors in ischemia models has been associated with limited success in the clinical setting, in part owing to the short lived effect of the injected cytokine. The use of a microparticle system could allow localized and sustained cytokine release and consequently a prolonged biological effect with induction of tissue revascularization. To assess the potential of $VEGF_{165}$ administered as continuous release in ischemic disease, we compared the effect of delivery of poly(lactic-co-glycolic acid) (PLGA) microparticles (MP) loaded with $VEGF_{165}$ with free VEGF or control empty microparticles in a rat model of ischemia-reperfusion. $VEGF_{165}$ loaded microparticles could be detected in the myocardium of the infarcted animals for more than a month after transplant and provided sustained delivery of active protein in vitro and in vivo. One month after treatment, an increase in angiogenesis (small caliber caveolin-1 positive vessels) and arteriogenesis (α-SMA positive vessels) was observed in animals treated with VEGF microparticles ($p<0.05$), but not in the empty-microparticles or free VEGF groups. Correlating with this data, a positive remodeling of the heart was also detected in the VEGF-microparticle group with a significantly greater LV wall thickness ($p<0.01$). In conclusion, PLGA microparticle is a feasible and promising cytokine delivery system for treatment of myocardial ischemia. This strategy could be scaled up and explored in pre-clinical and clinical studies.

Key words: angiogenesis; VEGF; PLGA microparticles; Controlled Release; myocardial infarction.

1. Introduction

Cardiovascular diseases remain the first cause of morbidity and mortality in the developed countries accounting for almost 30% of all deaths [1]. Despite recent evidence indicating that the heart is endowed with a regerative potential based on the presence of cardiac progenitors/stem cells, this is insufficient overall to prevent the development of cardiac failure after myocardial infarct in the majority of patients [2, 3]. While heart transplant remains the only curative option for patients with end-stage heart failure, new approaches such as gene (reviewed in [4, 5]) and stem cell therapy (reviewed in [6, 7]) or even the direct administration of pro-angiogenic growth factors have been explored in recent years [8, 9]. In the case of cell therapy, the current view suggests that stem cells contribute to cardiac repair through a paracrine effect associated with the release of growth factors rather than by directly contributing to tissue regeneration [10-14].

If the role of paracrine mechanisms is taken as a starting point, the administration of growth factors to promote tissue revascularization represents an attractive option that has been explored in animal models of limb ischemia or myocardial infarction. Pro-angiogenic cytokines such as VEGF (Vascular Endothelial Growth Factor), FGF (Fibroblast Growth Factor) or HGF (Hepatic Growth Factor) [15-18] have been administered either as natural recombinant human proteins or by gene transfer. However, although preclinical animal models and initial clinical trials suggested a beneficial effect [19-21], double-blinded clinical trials with large cohorts of patients failed to show efficacy [22-25]. These disappointing results were attributed, at least partially, to the short lived effect and high instability of the protein when injected as a bolus. On the other hand, gene delivery through naked plasmids or integrative viral

vectors was associated with low efficacy, or even with risk of genome integration, which hinders their use in the clinical setting [23, 25, 26].

An alternative approach is the development of biocompatible delivery systems, allowing for sustained and controlled release of growth factors that could prevent some of the problems described, and which would at the same time facilitate stable prolonged treatment in the damaged tissue (reviewed in [27]). To explore this possibility, we prepared poly(lactide-co-glycolide) (PLGA) microparticles (MP) containing the angiogenic cytokine $VEGF_{165}$ by water/oil/water (W/O/W) multiple emulsion solvent evaporation using the Total Recirculation One-Machine System (TROMS), a suitable technique for encapsulating proteins [27]. Next, we analyzed the vasculogenic effect of small-sized biodegradable and biocompatible VEGF-loaded microparticles in a rat model of myocardial infarction induced by ischemia reperfusion.

2. Materials and Methods

2.1 Materials

Recombinant human $VEGF_{165}$ (rhVEGF, Sf21-derived) was purchased from R&D Systems (Minneapolis, MN, USA). Quantikine VEGF ELISA kit was obtained from R&D Systems and used according manufacturer's instructions. PLGA with a monomer ratio (lactic acid/glycolic acid) of 50:50 Resomer® RG 503H (M_w: 34 kDa) was provided by Boehringer-Ingelheim (Ingelheim, Germany). Polyethylene glycol (PEG; M_w: 400), human serum albumin (HSA), bovine serum albumin (BSA) and sodium azide were provided by Sigma–Aldrich (Barcelona, Spain). Dichloromethane and acetone were obtained from Panreac Quimica S.A. (Barcelona, Spain). Poly(vinyl alcohol) (PVA) 88% hydrolyzed (M_w: 125,000) was obtained from Polysciences, Inc.

(Warington, USA). Rhodamine B isothiocyanate was from Sigma-Aldrich (Barcelona, Spain). A human iliac artery endothelial cell line (HIAE-101, ATCC, USA) was used in the bioactivity studies. Rabbit polyclonal anti-human VEGF-A (clone A-20, sc-152) was supplied by Santa Cruz Biotechnology (Santa Cruz, CA, USA). ECL™ anti-Rat IgG horseradish peroxidase-linked whole antibody was from Amersham Biosciences (Buckinghamshire, UK). Anti-caveolin-1α antibody was purchased from BD Biosciences Pharmingen (Heidelberg, Germany) and Alexa Fluor 594 goat conjugated anti mouse IgG was from Invitrogen (Eugene, OR, USA). Anti-alpha smooth muscle actin-Cy3 was provided by Sigma (St. Louis, MO, USA). Rabbit anti-caveolin-1 antibody was supplied by Cell Signaling Technology (Danvers, MA, USA) and donkey anti-rabbit FITC was from Jackson ImmunoResearch (West Grove, PA, USA).

2.2 Preparation of PLGA microparticles containing VEGF

rhVEGF$_{165}$-loaded microparticles were prepared by the solvent extraction/evaporation method using TROMS [27]. Briefly, 50 mg of PLGA were dissolved in 2 ml of a dichloromethane/acetone mixture. This polymer solution was injected into the inner aqueous phase (W_1) containing 35 μg of VEGF, 5 mg of HSA and 5 μl of PEG 400 dissolved in 200 μl of phosphate-buffered saline (PBS). Next, the previously formed inner emulsion (W_1/O) was recirculated through the system for 3 min under a turbulent flow regime. After this step, the first emulsion was injected into the outer aqueous phase (W_2), composed of 30 ml of a 0.5% PVA solution, resulting in a multiple emulsion (W_1/O/W_2), which was homogenized by circulation through the system for 4 min. The multiple emulsion was stirred for 3 h to allow solvent evaporation. Microparticles were washed three times with ultrapure water by

consecutive centrifugation at 4°C (20000 *g*, 10 min). Finally, the particles were resuspended in 1 ml of ultrapure water, frozen at -80°C, lyophilized (Genesis 12EL, Virtis) and stored at 4°C. For fluorescence-labeled microparticle formulation, rhodamine B isothiocyanate (0.5 mg/mL) was added to inner aqueous phase and microparticles were prepared as described.

2.3 Characterization of microparticles

2.3.1 Particle size analysis

Particle size and particle size distribution were measured by laser diffractometry using a Mastersizer® (Malvern Instruments, UK). A suitable amount of freeze-dried microparticles was resuspended in deionized water before measurement. The average particle size was expressed as the volume mean diameter in micrometers.

2.3.2 Determination of VEGF Encapsulation

Encapsulation efficiency was determined via extraction with dimethyl sulfoxide (DMSO). Freeze-dried loaded microparticles (2 mg, n = 3) were dissolved with 250 µl of DMSO as previously performed [27]. The amount of VEGF entrapped in the particles was measured using the Quantikine VEGF ELISA kit following the manufacturer's protocol.

VEGF content into microparticles was also quantified using Western blot analysis. After VEGF extraction from microparticles with DMSO, SDS-PAGE was performed onto 12% polyacrylamide gels and after electrophoresis the proteins were transferred onto nitrocellulose membranes. After 1 h blocking with 5% nonfat dried milk in TBS plus 0.05% Tween 20, nitrocellulose sheets were incubated overnight at 4

°C with primary rabbit antibodies against VEGF-A (A-20): sc-152 (diluted 1:2000). The binding of primary antibodies was performed by incubating membranes with horseradish peroxidase (HRP)-conjugated anti-rabbit secondary antibody (diluted 1:2000). Immunoreactive bands were, after several washes, visualized using LumiLight Plus western blotting substrate (Roche Diagnostics, Mannheim, Germany). Quantitative analysis of MP-extracted VEGF bands was performed by densitometry using Quantity One software (Bio-Rad Laboratories Inc., Munich, Germany). Sample values were quantified using a standard curve.

2.3.3 In vitro release of VEGF from PLGA microparticles

For determination of the cytokine release profile, 2 mg of VEGF-loaded microparticles (n = 3) were dispersed in 100 µl of PBS, pH 7.4, containing 0.1% BSA and microbiologically preserved with 0.02%$_{w/w}$ sodium azide. Incubation took place in rotating vials maintained at 37°C for 28 days. At defined time intervals (1 hour, 6 hours, 1, 2, 4, 7, 14, 21 and 28 days), sample tubes were centrifuged (25000 *g*, 15 min) and the supernatant was removed and frozen at −80°C. The removed solution was replaced with an equal volume of fresh medium. Supernatant protein content was determined by ELISA and western-blot assays.

2.3.4 Bioactivity of released VEGF

The bioactivity of the VEGF released from the microparticles was evaluated *in vitro* by determining the proliferative capacity of an endothelial cell line (HIAEC) after VEGF treatment. HIAECs were cultured in F12K media supplemented with 30 µg/mL endothelial cell growth supplement (ECGS), 10% fetal bovine serum, 1% heparin and

1% penicillin/streptomycin. In order to determine the endothelial cell proliferation capacity after VEGF stimulation, the HIAECs were plated into 24-well culture plates at a density of 1.25×10^3 cells/well and microparticles were placed in an upper chamber by using transwells (0.4 µm pore size, tissue culture treated polycarbonate membrane - Corning, USA). Cells were incubated for 3 or 7 days, with supernatant from non-loaded or VEGF-loaded microparticles, free VEGF (at 10 or 25 ng/mL), or medium alone as control. The number of viable cells in each experimental group was determined by ATP quantitation, which signals the presence of metabolically active cells by using the CellTiter-Glo® Luminescent Cell Viability Assay (Promega, USA). Moreover, in order to confirm the VEGF bioactivity, the capability of the released VEGF to activate its receptor (KDR) in the cultured HIAECs was evaluated by measuring KDR-tyrosine phosphorylation using an ELISA assay (DuoSet® IC for human phospho-VEGF R2/KDR, R&D Systems, Minneapolis, MN, USA).

2.4 In vivo experiments

2.4.1 Myocardial Infarction Model

The investigation conforms to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). All animal procedures were approved by the University of Navarra Institutional Committee on Care and Use of Laboratory Animals as well as the European Community Council Directive Ref. 86/609/EEC. A total of 30 female Sprague-Dawley rats (250-300 g) were obtained from Harlan-IBERICA (Barcelona, Spain). Animal experiments were carried out using a rat model of cardiac acute-reperfusion ischemia. Rats were initially anesthetized with 4% isoflurane in an

induction chamber. Prior to surgery, animals received analgesic drug ketoprofen 5 mg/Kg subcutaneously, fentanyl 0.15 mg/kg and heparin 0.1 mg/kg both administered by intraperitoneal route. The rats were then intubated and ventilated at 90 cycles/min (1.5-2% isoflurane was maintained for continuous anesthesia). A left thoracotomy through the fourth intercostal space was performed, and the left anterior descending (LAD) coronary artery was occluded 2-3 mm distal from its origin for 1 hour and then re-opened. The chest was then closed in layers and rats allowed to recover. Those rats with an ejection fraction bellow 55% (as determined by echocardiography) 2 days after myocardial infarction were included in the study.

2.4.2 Intramyocardial administration of microparticles

Four days after LAD coronary artery occlusion, 2 mg of VEGF-loaded microparticles or free VEGF (100 ng), or non-loaded microparticles (NL-MP) were injected with a 29-gauge needle into 4 regions in the border zone surrounding the infarct. Prior to injection, freeze-dried microparticles were dispersed in a sterile buffered solution consisting of 0.1% (w/v) carboxymethylcellulose, 0.8% (w/v) polysorbate 80 and 0.8% (w/v) mannitol in PBS, pH 7.4. All groups received the same volume of buffered solution (100 µl) and 6 animals were included in each group. The chest was closed and rats were allowed to recover.

2.4.3 PLGA-microparticles visualization in the myocardium

In order to evaluate the persistence of PLGA microparticles in the heart tissue, a group of infarcted animals was sacrificed 8 and 30 days after fluorescent-labelled microparticle administration (drug-free). Rhodamine B was used as a fluorescent

marker to localize the injected microparticles by fluorescent microscopy in the heart tissue.

2.5 Morphometric Study

Four weeks post-injection, animals were sacrificed and their hearts were collected for subsequent morphometric and histological analysis. After being harvested, the hearts were weighed and perfused-fixed in 4% paraformaldehyde at 4°C, and sliced in three 4-mm-thick segments from apex to base. The hearts were dehydrated in ethanol 70% at 4°C and embedded in paraffin. Sections (5 µm) were cut from each segment and stained with Sirius Red as previously described [28] to evaluate infarct wall thickness. These morphometric parameters were measured in images made with a 5× objective of Sirius Red-stained sections viewed with a Zeiss Axio Imager M1 microscope (Carl Zeiss AG, Oberkochen, Germany) and captured using an Axio Cam ICc3 video camera and Axiovision software (4.6.3.0 version). Fibrosis was measured in high power photographs within the infarct border as the percentage of collagen area (red) vs. total tissue area, using AnalySIS® software (Soft Imaging System GmbH, Münster, Germany).

2.6 Histological Study

For the analysis of capillary density (capillaries/mm^2), 9 sections per heart were stained with an anti-caveolin-1α antibody (diluted 1:50) and 2 peri-infarct and 2 intra-infarct images per section were analyzed. Secondary antibody was Alexa Fluor 594 goat conjugated anti mouse IgG (diluted 1:100). The arteriolar density and arteriolar area were quantified in the same way after staining with anti-alpha smooth muscle actin-Cy3

(α-SMA, diluted 1:500) in the following sections. For vessel counting, images were acquired using the Axio Cam MR3 video camera at 20× connected to the Zeiss Axio Imager M1 microscope equipped with epifluorescence optics. Digital images were analyzed using MatLab® software platform (Mathworks Inc., Natick, MA, USA). Also, double immunostaining with anti-alpha smooth muscle actin-Cy3 and rabbit anti-caveolin-1 (diluted 1:125) was performed. Secondary antibody was donkey anti-rabbit FITC (diluted 1:200) and nucleus were stained with TOPRO-3 (diluted 1:50 in PBS-glycerol). For confocal microscopy, a LSM 510 META (Carl Zeiss, Minneapolis, USA) microscope was utilized.

2.7 Statistical analysis

Results are expressed as mean ± SEM. Statistics were calculated with SPSS computer software for Windows (version 15.0, SPSS Inc, Chicago, Ill). Non-parametric statistical analyses were used when values were not normally distributed. The differences among the groups were first evaluated using the Kruskall–Wallis test, followed by Mann–Whitney U-test comparing individual groups where necessary. The differences among the groups were assessed by ANOVA with a Tukey post hoc correction when the measured values were normally distributed. Shapiro-Wilk test was used to justify the use of a parametric test. A value of $p<0.05$ was considered statistically significant.

3. Results

3.1 Characterization of PLGA-microparticles, $VEGF_{165}$ release and bioactivity

VEGF-loaded microparticles (VEGF-MP) were prepared with a diameter of 5.1±1.3 µm (Fig.1A). The total amount of loaded VEGF was 0.58 µg per mg of polymer, which corresponds to an encapsulation efficiency of 83.8±6.6% determined by ELISA and confirmed by Western Blot analysis. The yield of the microencapsulation process was over 80%.

The *in vitro* release kinetics was performed in PBS (pH 7.4) at 37 ºC for 28 days. VEGF released within the first 6 hours (*burst* effect) was 10±1%, followed by a phase of sustained release of the cytokine with almost 75% of VEGF being released within 28 days (Fig.1B).

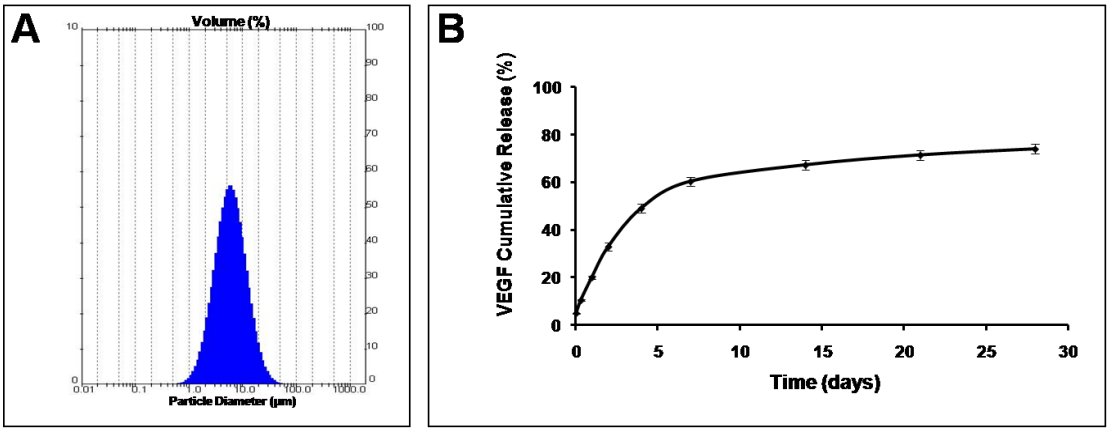

Figure 1: Microparticle characterization and Release Profile. A. Representative particle size distribution for PLGA microparticles prepared by TROMS employed in the *in vivo* studies. **B.** *In vitro* release of VEGF from PLGA microparticles.

The bioactivity of the encapsulated $VEGF_{165}$ released from the microparticles was examined by determining its capacity to induce proliferation of endothelial cells (Human Iliac Artery Endothelial Cells HIAEC). VEGF-MP (10 or 25 ng/mL) induced a

2-3 fold increase in proliferation of HIAEC in comparison with control (no cytokine) or non-loaded MP (NL-MP) after 3 and 7 days in culture ($p<0.01$). This increase was similar to that observed when HIAEC cells were cultured with daily addition of free VEGF at doses of 10 or 25ng/mL (Fig.2A). In addition, bioactivity of the VEGF-MP was further determined by quantifying the tyrosine phosphorylation of the VEGF receptor KDR. Similar levels of stimulation in the HIAEC cells treated either with the free-VEGF or the VEGF-MP was detected, confirming the bioactivity of the released protein (Fig.2B).

3.2 ***Microparticle visualization in the heart tissue***

In order to confirm the capacity of the microparticles to remain in the myocardium for a prolonged period of time -a requirement for sustained cytokine treatment-, the fate of the particles after *in vivo* administration was assessed. Rhodamine-labeled microparticles were injected into the peri-infarcted area of the myocardium and animals were sacrificed at 1 and 4 weeks. The fluorescent-labeled microparticles were visible by fluorescence microscopy for up to a month post-implantation (Fig.3A-E). Furthermore, partial degradation of the particles was observed after one month, indicating the biodegradable nature of the co-polymer (Fig.3D,E).

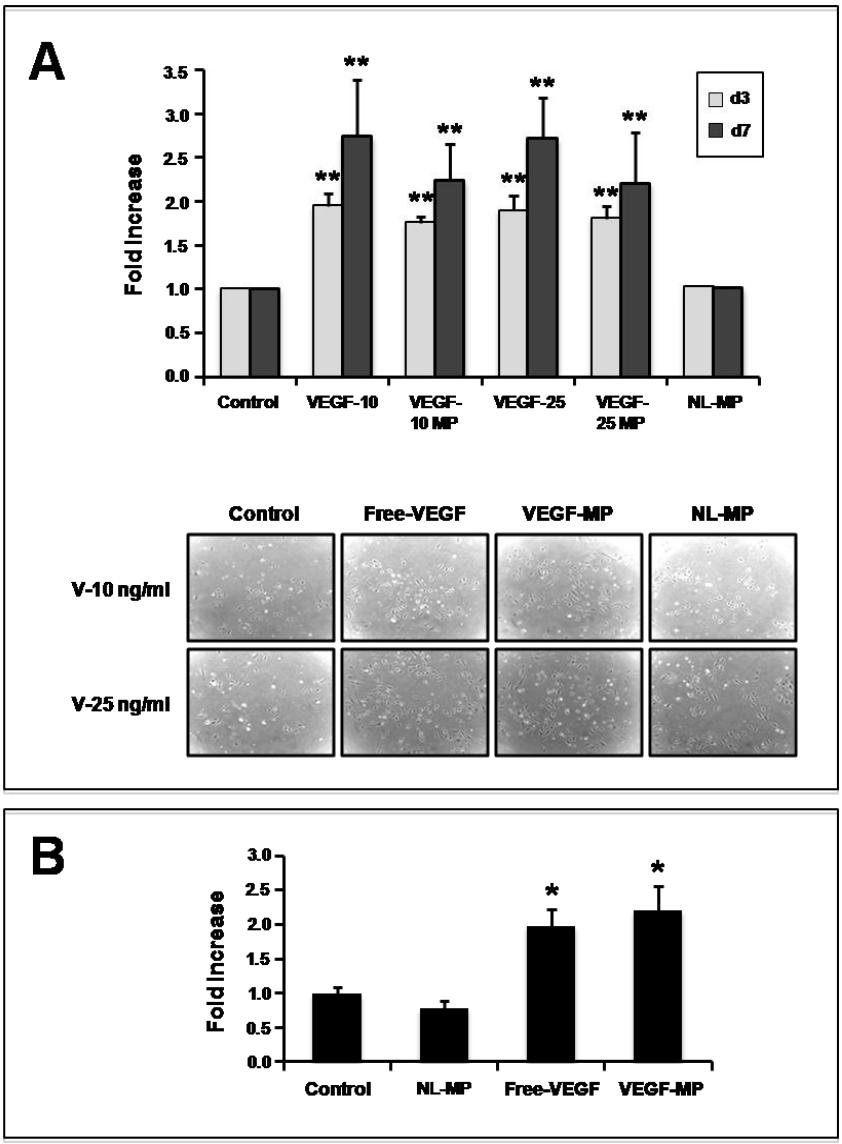

Figure 2: Bioactivity of VEGF-MP. **A.** Proliferation of HIAEC cells was induced by free VEGF at 10ng/mL (VEGF-10) or 25ng/mL (VEGF-25) or VEGF-MP at the same concentration and compared to culture medium alone (control) for 3 and 7 days. Non-loaded microparticles (NL-MP) did not induce cell proliferation. Representative pictures of HIAECs density 7 days after treatment are shown. **B.** KDR activation in HIAECs induced by VEGF stimulation (free or encapsulated at 10ng/mL) and NL-MP (y axis represents fold increase *versus* non treated group)**.** **P*<0.05 and ***P*<0.01.

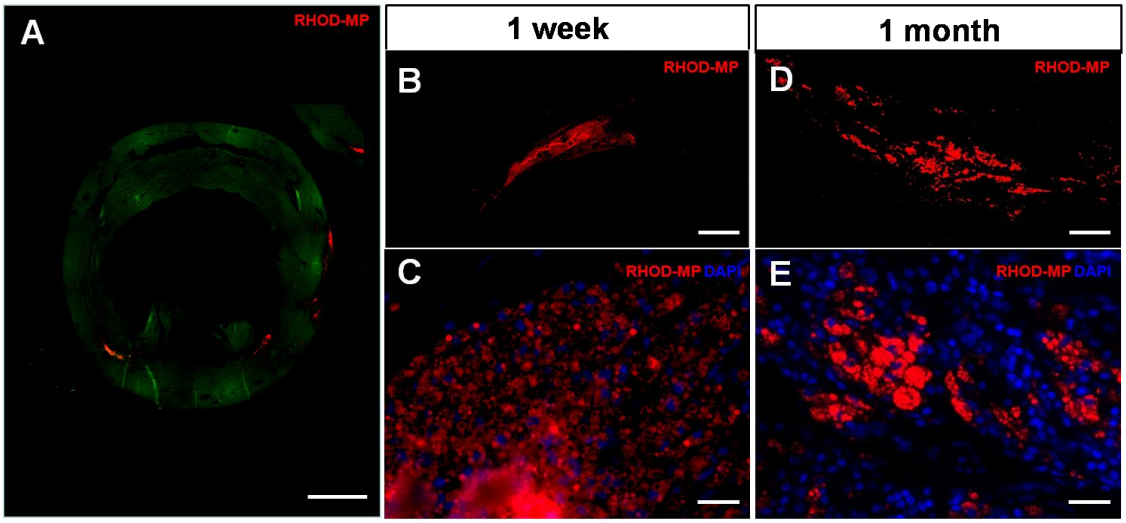

Figure 3: (A) PLGA-microparticles visualization in the heart tissue. A. Microscopy fluorescence visualization of a heart cross-section after fluorescent-labelled microparticle administration**. B-E.** Rhodamine-labeled-microparticles distribution 1 week **(B,C)** and 1 month **(D,E)** after injection. Nuclear staining was performed with DAPI (blue). Scale bars: 500 µm **(A)**, 100 µm **(B,D)** and 20 µm **(C,E)**.

3.3 Vasculogenic and tissue remodeling effect of treatment with VEGF-MP

Administration of VEGF-MP in infarcted rats was associated with a statistically significant increase in the number of capillaries in the infarct and peri-infarct areas of the injured hearts in comparison with the control group (non-loaded microparticles: NL-MP) or with animals treated with free VEGF (NL-MP: 579.5±33.8; VEGF-MP: 704.9±31.75, P<0.05; Free-VEGF: 571.6±37.3, P=NS, capillaries/mm^2) (Fig.4A). Treatment with VEGF-MP not only induced a significant increase in small caliber vessels, but also in the number of arterioles (α-SMA coated vessels). This effect was only detected in the hearts treated with the VEGF-MP and not in the hearts injected with the free cytokine, which showed a similar vessel density to animals treated with non-loaded MP (NL-MP: 70±6.7; VEGF-MP: 95±8.9, P<0.05; Free-VEGF: 55±4.5, P=NS, arterioles/mm^2) (Fig.4B). In line with these results, the area occupied by α-SMA-positive-vessels (µm^2) was significantly increased in animals treated with VEGF-MP (NL-MP: 3347±183; VEGF-MP: 6590±764, P<0.001; Free-VEGF: 2170±328, P=NS).

No hemangioma formation or leaking vessels were detected in the VEGF-MP group in the analysis of hematoxylin-eosin stained sections, confocal 3D analysis of caveolin-1^+ stained vessels -where non-leaking vessels were detected- (Fig.5A,B), and confocal analysis of caveolin-1^+/α-SMA double-stained vessels, which showed a tightly association between the endothelial and mural cell layers (Fig.5C-E).

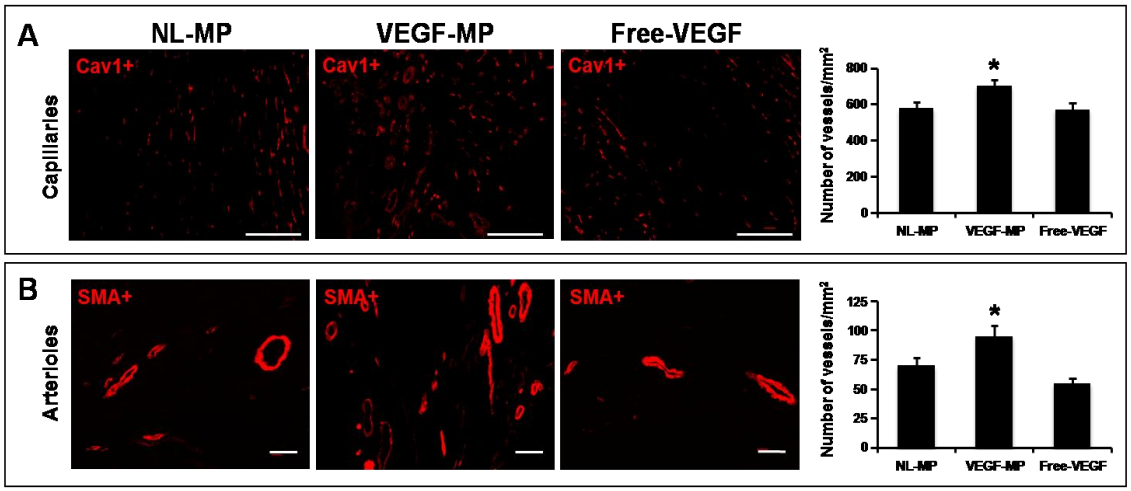

Figure 4: *In vivo* effects of VEGF-MP. Capillary **(A)** and arteriole **(B)** densities were determined by quantification of the small caliber (<15µm) caveolin-1-positive capillaries/mm^2 and α-SMA-positive vessels/mm^2 in the infarcted and peri-infarcted areas, 1 month after administration of non-loaded MP (NL-MP) (control group), VEGF-MP or free-VEGF. Representative images for caveolin-1α and α-SMA immunofluorescence stainings are shown. A significant increase in capillary and arteriole densities was determined in the hearts injected with VEGF-MP in comparison with the control group (*P<0.05). No significant increase was detected in the free-VEGF group. Data are presented as mean ± SEM. Scale bars: 50 µm.

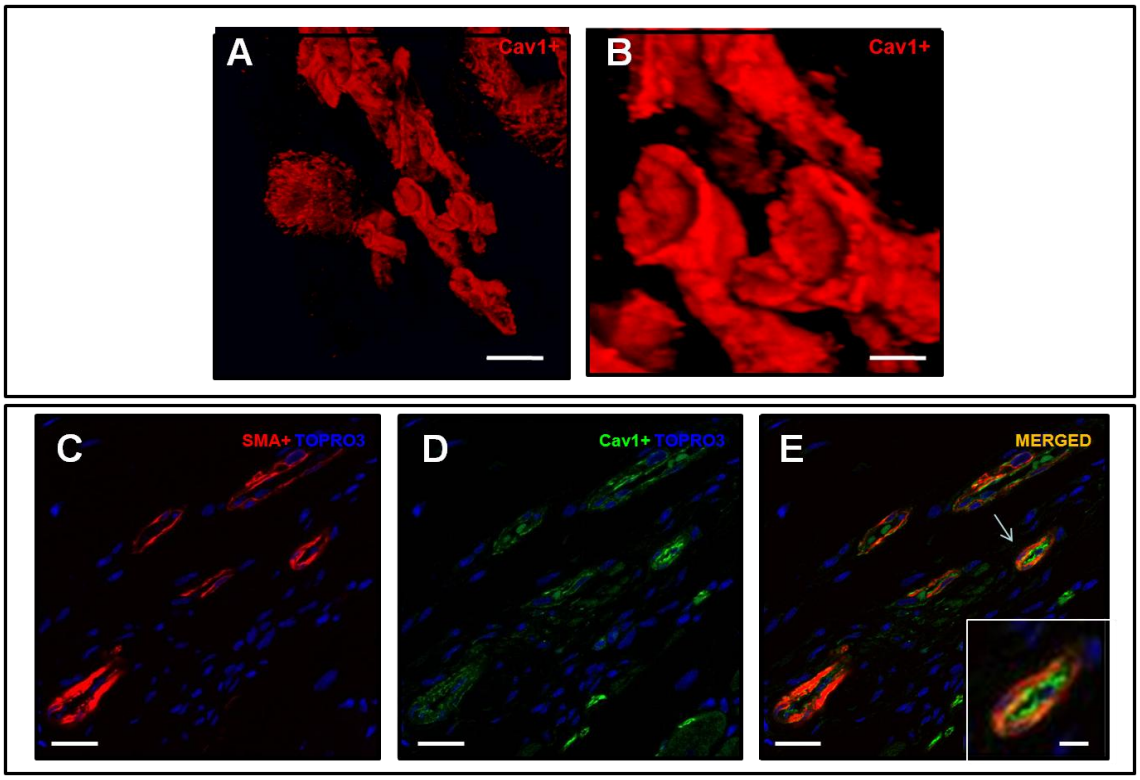

Figure 5: Vessel structure. A,B. Tri-dimensional views of caveolin-1-positive vessels in the VEGF-MP group. Note that the vessels display a regular endothelial structure. **C-E.** Representative pictures of αSMA-Cy3 (red) and Caveolin-1 (green) double immunostained vessels in VEGF-MP-treated heart sections, showing tightly contact between the smooth muscle and the endothelial cell layers. Nuclear staining was performed with TOPRO-3 (blue). Scale bars: 100 µm (**A**), 400 µm (**B**), 20 µm (**C-E**), 5 µm (**E**, insert).

The increased revascularization of the tissue translated into a beneficial effect in the remodeling processes, with a significantly greater thickness of the left ventricle wall in the VEGF-MP treated animals in comparison with the control group (NL-MP: 1.07±0.02 mm; VEGF-MP: 1.30±0.05 mm (P<0.01); Free-VEGF: 1.07±0.10 mm (P=NS)) (Fig.6), suggesting a potential benefit related to the administration of VEGF through a sustained release system in comparison with injection of the free cytokine.

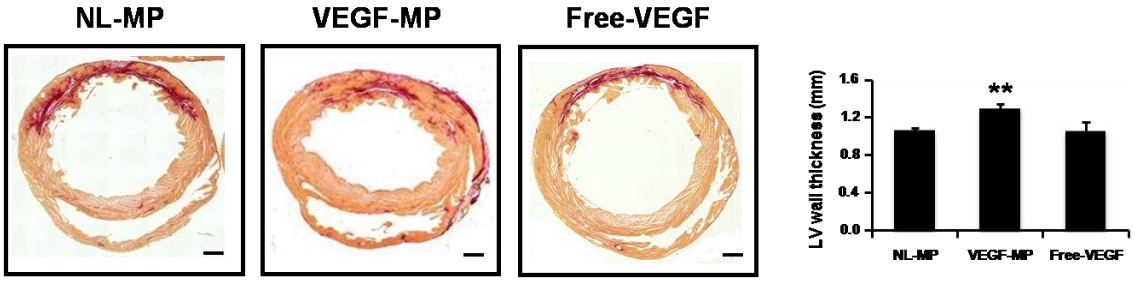

Figure 6: Heart remodeling. Representative Sirius red stained heart sections show greater thickness of the LV wall of the VEGF-MP group in comparison with the NL-MP (control group) (***P*<0.01). No significant increase was detected in the free-VEGF treated group. Scale bars: 1 mm.

4. Discussion

In patients with myocardial ischemia, therapeutic angiogenesis by direct delivery of the VEGF protein or using gene therapy approaches has not yielded the expected results in clinical practice [29]. Some important limitations, such as the short half-life of the protein, as well as the relatively short effect of naked plasmids or adenovirus transfection, seemed to be responsible, at least partially, for the lack of success. Alternative strategies that allow a local, sustained delivery of VEGF or other angiogenic cytokines are worth exploring in order to induce a therapeutic effect. The development of PLGA microparticles loaded with VEGF shown in our study fulfills many of the requirements for a potentially successful therapy like the possibility of being readily administered and their permanence in the myocardium for at least 30 days, being capable of a sustained delivery of active cytokine during that period of time.

Therefore, treatment with VEGF-MP was associated with the expected biological effect: increased angiogenesis and arteriogenesis in an acute ischemia-reperfusion model. Other delivery systems based on the use of hydrogels, liposomes, nanoparticle-fibrin complex, collagen-bound proteins or polymer scaffolds with VEGF or other cytokines have also been explored, showing an angiogenic effect after sustained cytokine treatment [30-34]. However, in general the control of release rate of growth

factors from hydrogels is difficult and a strong initial burst release is generally observed which has been associated with severe side effects, such as hypotension [30]. Interestingly, targeted delivery has recently been achieved by anti-P-selectin-conjugated liposomes, which induced an increase in tissue vascularization and an improvement in the cardiac function [31]. Unfortunately, the need for very early delivery (a down-regulation of the receptor in the infarcted area occurs 24h after the ischemia) [35] represents a challenge for its therapeutic use in patients.

VEGF is known to be a potent key regulator of blood vessel formation during both angiogenesis and vasculogenesis (reviewed in [36]). Endothelial cells are activated by VEGF, which results in increased vessel permeability, cell migration and proliferation. Indeed, local high levels of VEGF may result in deleterious effects including the formation of irregularly shaped sac-like vessels associated with massive and highly disruptive edema [37] or even formation of endothelial cell-derived intramural vascular tumors [38]. The sustained controlled release provided by the PLGA particles seems to circumvent these potential problems, as none were found in our study. The positive effects of VEGF-MP on the induction of angiogenesis were not observed in animals treated with NL-MP or even with free-VEGF, demonstrating the greater effect of long-term-release VEGF. As it has been previously demonstrated, a short VEGF stimulus, if it is not sustained, is associated with the regression of the newly formed vessels when discontinued [37].

Treatment with VEGF-MP induced not only an increase in capillaries, but also in vessels of greater caliber, suggesting that prolonged release of VEGF indirectly promotes arteriogenesis by stabilizing the vessels through pericyte and/or smooth muscle recruitment and proliferation (reviewed in [39]). It has been suggested that

VEGF can induce the proliferation of local pericytes by stimulating endothelial cells to express PDGF (platelet-derived growth factor) [40], which exerts a chemotactic and differentiation effect in pericytes. Furthermore, the presence of the VEGF receptor in pericytes has also been shown [41], implying that VEGF can act directly on the pericytes by stimulating migration and proliferation. Interestingly, sustained VEGF-MP treatment induced an increase in the number of arteriolar vessels, favoring tissue irrigation [42] and consequently, rescuing the tissue areas at risk. It is possible that preexisting capillaries could undergo enlargement and/or fusion and recruit a smooth muscle cell coat as a consequence of the sustained VEGF-stimulation [42-44]. On the other hand, the presence of VEGF receptors in cardiomyocytes and their association with a protective effect has been reported [45], which could be responsible, together with the revascularization effect, for the rescue of the cardiac tissue, which translated in a positive remodeling of the heart.

Timing and combination of cytokine administration is an important issue. VEGF-MP were administered alone and shortly after the ischemic event. From the results of this study and others where cytokine was given immediately after myocardial infarction, it seems that very early injection of the cytokine might favor its therapeutic benefit by rescuing hibernating cardiac tissue. On the other hand, the combination of other cytokines that contribute to vessel maturation such as PDGF, TGFβ, FGF, or angiopoietins or even combination of cytokines and (stem) cells could induce a stronger beneficial effect [46-48]. Thus, in example, it has been shown, for example, that subcutaneous implantation of a polymer scaffold that released $VEGF_{165}$ and PDGF-BB induced rapid formation of a mature vascular network [49].

Conclusions

In summary, we have demonstrated that a single cytokine, VEGF, could exert not only an angiogenic but also an arteriogenic effect when delivered *in vivo* in a sustained manner, which translates into positive remodeling of the heart. Moreover, the use of microparticles allows a dose-controlled release of the protein that can be easily and safely translated to patients.

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CHAPTER 3

CHAPTER 3

Controlled delivery of fibroblast growth factor-1 and neuregulin-1 from biodegradable microparticles promotes cardiac repair in a rat myocardial infarction model

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ABSTRACT

Myocardial infarction (MI) is a major health concern worldwide, and therefore, extensive research has been performed to find new treatments. Acidic fibroblast growth factor (FGF-1) and neuregulin-1 (NRG-1) have been identified as factors involved in cardiac repair after MI. However, the therapeutic value of these growth factors has important limitations *in vivo,* related to their short-lived effect and high instability after systemic administration. To circumvent these limitations, FGF-1 and NRG-1 were encapsulated into poly-lactide-*co*-glycolide (PLGA) microparticles (MP), which released the bioactive growth factors in a sustained manner for up to 28 days *in vitro*. The ability of FGF-1 and/or NRG-1 MP to promote cardiac regeneration was evaluated in a rat model of MI. Three months after treatment, a cardiac function improvement was detected in the rats treated with FGF1-MP (16.7 ± 4.9%, $P<0.05$), NRG1-MP (18.0 ± 5.7%, $P<0.05$) or FGF1/NRG1-MP (13.0 ± 1.9%, $P<0.05$) in comparison with the non-loaded (NL-MP) control group (1.1 ± 3.6%). In addition, a positive cardiac remodeling with a smaller infarct size and lower fibrosis degree, an induction of tissue revascularization and cardiomyocyte proliferation was detected. Also, recruitment of c-Kit^+ progenitor cells towards the ischemic myocardium under stimulation of FGF-1 and NRG-1 delivered from the MP was detected. Collectively, these results demonstrate the ability of PLGA-MP to deliver efficiently FGF-1 and NRG-1, which promoted myocardial regeneration by distinct mechanisms of cardiac repair after MI.

Key words:FGF-1, NRG-1, PLGA microparticles, myocardial infarction, cardiac repair

1. Introduction

Ischemic heart disease (IHD) is the leading cause of morbidity and mortality worldwide [1, 2]. Usually, IHD occurs when a coronary artery narrows (frequently as a result of atherosclerosis) and heart blood supply is insufficient, resulting in angina, myocardial infarction (MI) and heart failure [3]. Current therapies include pharmacological treatments, percutaneous intervention and surgery. These approaches can mitigate the symptoms but are not able to regenerate the tissue or to restore the heart function, being the heart transplant the only curative option for patients with end-stage heart failure. New alternatives such as gene (reviewed in [4]) and stem cell therapy (reviewed in [5]) or even direct administration of pro-angiogenic cytokines have been explored in the recent years [6]. In the case of growth factor-based therapy, although pre-clinical studies and initial clinical trials suggested a beneficial effect [7-9], double-blinded clinical trials with large cohorts of patients failed to show efficacy [10-12]. Issues like the choice of growth factor agent, monotherapy instead of combinatorial therapy and timing of growth factor delivery might have been responsible for the negative results. Also, the short circulating half-life and high instability of growth factors when injected as a bolus represent a great limitation. In this context, new strategies such as injectable biocompatible and biodegradable slow-release polymers could overcome these shortcomings, allowing a sustained and controlled release of factors and, in that way, a much more localized, stable and prolonged treatment of the damaged tissue [13]. Poly(lactic-*co*-glycolic acid) (PLGA) microparticles (MP) have already demonstrated their potential for growth factor delivery [14-16]. In fact, PLGA is an attractive biomaterial because of its excellent biocompatibility, high safety profile and FDA approval for usage in drug delivery [17]. We previously demonstrated the

efficacy of treating the infarcted heart with PLGA MP loaded with vascular endothelial growth factor (VEGF), which induced neovascularization and a positive remodeling of the infarcted heart in contrast with the free cytokine treatment, that did not induce any effect [18].

Furthermore, the majority of experimental studies and clinical settings have explored the angiogenic potential of cytokines to promote tissue revascularization, and the use of other cytokines involved in other mechanisms such as stem cell mobilization and homing, cardiac differentiation and proliferation and direct cardioprotection has not been so intensively approached (reviewed in [19]).

Interestingly, *in vitro* studies have shown that adult cardiomyocytes do not proliferate under resting conditions but may proliferate in response to extracellular mitogens such as periostin [20], acidic fibroblast growth factor (FGF-1) [21] and neuregulin-1 (NRG-1) [22]. These evidences support the new paradigm that the heart might be capable of repair and regrowth in response to extracellular mitogens (reviewed in [23]). In fact, neuregulins play crucial roles in the adult cardiovascular system by inducing sarcomere structure organization, cell integrity, cell-cell adhesion [24], cell survival [25] and angiogenesis [26]. Also, FGF-1 plays a pivotal role in the regulation of cardiac remodeling by exerting a protective and proliferative effect after myocardial infarction [27-30].

In this work, we have analyzed the beneficial potential of biodegradable and biocompatible PLGA-MP containing FGF-1 and NRG-1. A rat model of acute MI was performed for therapeutic evaluation of both factors.

2. Materials and methods

2.1 Materials

Recombinant human FGF-1 and NRG-1 were supplied from ImmunoTools GmbH (Friesoythe, Germany). PLGA with a monomer ratio (lactic acid/ glycolic acid) of 50:50 Resomer® RG 503H (M_w: 34 kDa) was provided by Boehringer-Ingelheim (Ingelheim, Germany). Polyethylene glycol (PEG; M_w: 400), human serum albumin (HSA), bovine serum albumin (BSA), dimethylsulfoxide (DMSO) and sodium azide were provided by Sigma-Aldrich (Barcelona, Spain). Dichloromethane and acetone were obtained from Panreac Quimica S.A. (Barcelona, Spain). Poly(vinyl alcohol) (PVA) 88% hydrolyzed (M_w: 125,000) was obtained from Polysciences, Inc. (Warington, USA). Murine HL-1 cardiomyocyte-cell line (kindly donated by Dr. Claycomb, Louisiana State University Medical Center, USA) was used in the *in vitro* assays. Claycomb medium was provided by SAFC Biosciences (Lenexa, KS, USA) and 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H tetrazolium (MTS) was purchased from Promega (Madison, USA). Rabbit anti-human FGF-1 antibody (ab9588) was supplied by Abcam (Cambridge, UK). Goat polyclonal anti-human NRG-1 antibody (sc-1793) and horseradish-peroxidase-conjugated donkey anti-goat IgG (sc-2020) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). ECL™ anti-rat IgG horseradish peroxidase-linked whole antibody was from Amersham Biosciences (Buckinghamshire, UK). Anti-alpha smooth muscle actin-Cy3 was provided Sigma (St. Louis, MO, USA). Rabbit polyclonal anti-human c-Kit antibody (A4502) was supplied from Dako (Carpinteria, CA, USA). Ki-67 antibody (RM9106) was purchased from Thermo Fisher Scientific (Fremont, CA, USA) and cardiac troponin I antibody (ab19615) was from Abcam (Cambridge, UK).

2.2 Culture of HL-1 cells

Murine HL-1 cardiomyocyte-cell line has proven to be useful for studying many aspects of cardiac biology *in vitro* [31]. We cultured HL-1 cells for further experiments in order to assess the effects of FGF-1 and NRG-1 treatment on viability and survival of these cells. HL-1 cells were cultured in gelatinized T-25 cell culture flasks and allowed to grow in Claycomb medium supplemented with 10% fetal bovine serum, 1% L-glutamine and 1% penicillin/streptomycin at 37°C under 5% CO_2/95% air. Culture media were changed every 2-3 days and the cells were subcultured when 80–90% confluency was achieved.

2.3 Cardiomyocyte proliferation assay

For cell proliferation assay, HL-1 cells were seeded in gelatinized 96-well tissue culture at a density of 1.25×10^3 cells/well. After 24 h, medium was removed and the cells were incubated with different concentrations of FGF-1 and/or NRG-1 (at 1, 10, 25 and/or 100 ng/ml), or medium alone as control. Claycomb medium supplementation was modified by reducing the fetal bovine serum to 5% in the media. Three days after incubation, MTS assay was performed to quantify the viable cells.

2.4 Apoptosis Assays

Gelatinized 96-well tissue culture plates were inoculated with HL-1 cells at a density of 5×10^3 cells/well. After 24 h, medium was removed and the cells were washed with PBS and treated with different concentrations of FGF-1 and/or NRG-1 (at 1, 10 and/or 100 ng/ml) in serum-free Claycomb medium at 0.5% O_2 for 2 days. Control cells were left untreated. Apoptosis was firstly assessed using a quantitative nucleosome

ELISA (Cell Death Detection ELISA kit, Roche) to detect DNA fragmentation generated in HL-1 apoptotic cardiomyocytes.

A second assay consisted of caspase-3/7 activity measure. Briefly, HL-1 cells were seeded and treated under hypoxia conditions as described above. After treatment, cells were inoculated with Apo-ONE® caspase-3/7 reagent (Promega) containing Z-DEVD-R110, a caspase substrate known to be essentially cleaved by caspase-3 or caspase-7. Next, samples were incubated at 37°C for 1 h. Serum-free Claycomb medium was used as a blank. The fluorescence signal was recorded using a spectrofluorometer at the detection/emission wavelength pair of 499 nm/521 nm.

2.5 Preparation and characterization of growth factor-loaded PLGA microparticles

FGF-1 and NRG-1 were separately encapsulated into PLGA microparticles by solvent extraction/evaporation method using the Total Recirculation One-Machine System (TROMS), as previously described [18]. Non-loaded microparticles were also prepared similarly to loaded ones, but without growth factors in the formulation. Also, fluorescent-labeled microparticles (growth factor-free) were prepared with rhodamine B isothiocyanate.

Particle size and and size distribution of the microparticles were measured by laser diffractometry using a Mastersizer® (Malvern Instruments, UK). The morphology of the particles was characterized by scanning electron microscopy (SEM). Briefly, the lyophilized microparticles were mounted on carbon conductive disks attached to aluminum stubs. Samples were then coated with gold to a 16-mm thickness (Emitech K550 equipment). Microparticles were randomly scanned using SEM (Zeiss DSM 940A, Germany) and photomicrographs were taken.

2.6 Encapsulation efficiencies of FGF-1 and NRG-1 into PLGA microparticles

Both FGF-1 and NRG-1 content into microparticles was separately determined by western blot. After protein extraction from microparticles with DMSO, SDS-PAGE was performed onto 12% or 16% polyacrylamide gels and after electrophoresis the proteins were transferred onto nitrocellulose membranes. After 1 h blocking with 5% nonfat dried milk in TBS plus 0.05% Tween 20 (TBST), nitrocellulose sheets were incubated with primary rabbit antibodies against FGF-1 (diluted 1:2000) or goat antibodies against NRG-1 (diluted 1:50). Incubation took place in a shaking platform overnight at 4 °C under slow shaking. The binding of primary antibodies was performed by incubating membranes with horseradish peroxidase (HRP)-conjugated anti-rabbit or anti-goat secondary antibodies (diluted 1:2000). After several washes with TBST, immunoreactive bands were visualized using LumiLight Plus® western blotting substrate (Roche Diagnostics, Mannheim, Germany). For quantification, blots were digitally scanned and the density of the microparticle-extracted FGF-1 or NRG-1 bands was determined using Quantity One software (Bio-Rad Laboratories Inc., Munich, Germany). Sample values were quantified using a blotting standard curve with known amounts of FGF-1 or NRG-1.

2.7 In vitro release of FGF-1 and NRG-1 from PLGA microparticles

Cumulative release kinetics was conducted to determine the *in vitro* growth factor release profiles from the microparticles. Briefly, 2 mg of dry FGF1-MP or 3 mg of NRG1-MP (n=3) were suspended in 100 µl of release buffer (PBS, pH 7.4 with 0.1% BSA and 0.02% sodium azide). Incubation took place in rotating vials maintained at 37°C for 28 days. At defined time intervals (1 h, 4 h, 8 h, 1, 2, 3, 7, 14, 21 and 28 days),

sample tubes were centrifuged (25,000×g, 15 min) and the supernatant was removed and frozen at −80 °C until it was analyzed. The removed solution was replaced with an equal volume of fresh release buffer to maintain sink conditions. Supernatant protein content was determined by western blot assays.

2.8 Bioactivity of released growth factors

HL-1 cells were cultured in Claycomb medium supplemented with 10% fetal bovine serum, 1% L-glutamine and 1% penicillin/streptomycin. In order to quantify the stimulation of cell proliferation by microencapsulated growth factors, gelatinized 96-well tissue culture plates were inoculated with 1.25×10^3 cells/well. After 24 h, medium was removed and the cells were incubated with supernatant from non-loaded or growth factor-loaded microparticles, free growth factors (at 100 ng/ml), or medium alone as control. Claycomb medium supplementation was modified for these experiments by reducing the fetal bovine serum to 5% in the media. Three days after incubation, cell growth–promoting activity was detected by MTS assay.

2.9 Animal experiments

2.9.1 Rat myocardial infarction model

A total of 70 female Sprague-Dawley rats (Harlan-IBERICA, Spain) underwent permanent occlusion of the left anterior descending coronary artery (LAD), as previously described [32]. Briefly, rats were anesthetized with 4% isoflurane in an induction chamber and supported with a mechanical ventilator. Prior to surgery, animals received analgesic drug ketoprofen 5 mg/Kg subcutaneously and fentanyl 0.15 mg/kg by intraperitoneal route. The rats were then intubated and 1.5–2% isoflurane was

maintained for continuous anesthesia. The heart was accessed through a left thoracotomy through the fourth intercostal space, and the left anterior descending (LAD) coronary artery was permanently occluded 2–3 mm distal from its origin. The chest was then closed in layers and rats allowed to recover on a heating pad. Among surviving animals (n = 54), only those with a left ventricular ejection fraction (LVEF) below 50% (n = 40) 2 days post-MI were included in the study.

2.9.2 Intramyocardial administration of microparticles

Four days post-MI, rats were placed into four injection groups and the chest reopened. Two milligrams of FGF1-loaded microparticles (FGF1-MP, 1740 ng of FGF-1) or NRG1-loaded microparticles (NRG1-MP, 1300 ng of NRG-1) or a combination of microparticles loaded with the same doses of FGF-1 and NRG-1 (FGF1/NRG1-MP) or control non-loaded microparticles (NL-MP) were injected with a 29-gauge needle into 4 regions in the border zone surrounding the infarct. Prior to injection, freeze-dried microparticles were dispersed in a sterile buffered solution consisting of 0.1% (w/v) carboxymethylcellulose, 0.8% (w/v) polysorbate 80 and 0.8% (w/v) mannitol in PBS, pH 7.4. All groups received the same volume of buffered solution (80 μl) and 10 animals were included in each group. The chest was closed and rats were allowed to recover on heating pad. All animal procedures were approved by the University of Navarra Institutional Committee on Care and Use of Laboratory Animals as well as the European Community Council Directive Ref. 86/609/EEC.

2.9.3 Long-term retention of microparticles in the heart tissue

To determine whether microparticles were retained in the heart for a prolonged period of time, we developed fluorescent-labeled microparticles (factor-free). A group of infarcted animals was sacrificed 30, 60 and 90 days after rhodamine-loaded microparticles administration. Rhodamine B was used as a fluorescent marker to localize the injected microparticles by fluorescent microscopy in the heart tissue.

2.9.4 Echocardiography

Left ventricular (LV) function was studied in isoflurane anesthetized animals using a Sonos 4500 ultrasound system (Philips) with a 12 MHz linear array transducer and Doppler measurement. For measurement of end systolic and diastolic volumes and diameters, LV ejection fraction (LVEF) and LV mass, a parasternal short-axis view was used to obtain 2-dimensional and M-mode images. LV mass, volumes and diameters were normalized for body weight. Baseline echocardiography was performed 2 days after myocardial infarction. Only animals with LVEF $\leq$ 50% were included in the study. Three months after treatment, echocardiography was performed. All measurements were taken by a blinded observer. Animals were sacrificed and their hearts collected for subsequent morphometric and histological studies.

2.10 Morphometric study

Three months post-injection, the hearts were arrested with $CdCl_2$, perfused-fixed in 4% paraformaldehyde at 4 °C, and sliced in three 4-mm-thick segments from apex to base. The hearts were dehydrated in ethanol 70% at 4°C and embedded in paraffin. Sections (5 µm) were cut from each segment and stained with Sirius Red. The degree of

fibrosis, infarct size and LV wall thickness were measured in images made with a 5× objective of Sirius Red-stained sections viewed with a Zeiss Axio Imager M1 microscope (Carl Zeiss AG, Oberkochen, Germany) and captured using an Axio Cam ICc3 video camera and Axiovision software (4.6.3.0 version). Infarct size was assessed as the mean percentage of the infarcted area vs. the total LV area, and fibrosis was measured in high power photographs within the infarct border as the percentage of collagen area (red) vs. total tissue area, using AnalySIS® software (Soft Imaging System GmbH, Münster, Germany).

2.11 Vascular density

Immunostaining with anti-alpha smooth muscle actin-Cy3 (diluted 1:500) was performed. The vessels positive for alpha smooth muscle actin (α-SMA) were counted in infarct and peri-infarct zones. Four intra-infarct and four peri-infarct images per section were randomly selected and counted from 6 sections from each animal. Images were acquired using the Axio Cam MR3 video camera at 20× connected to the Zeiss Axio Imager M1 microscope equipped with epifluorescence optics. Digital images were analyzed using MatLab® software platform (Mathworks Inc., Natick, MA, USA). Arteriolar density was expressed as the number of α-SMA-positive-vessels per mm^2. The area occupied by α-SMA-positive-vessels (μm^2) was also determined.

2.12 Detection of apoptosis by TUNEL

Tissue sections were deparaffinized and pretreated with 0.1 M citrate buffer (pH 6) and microwave irradiation for 5 min. Terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling (TUNEL) was employed using an *in situ* cell death detection kit

with TMR Red (Roche Applied Science) to detect apoptotic cells. TUNEL-positive nuclei and total nuclei were counted from the border zone of the infarction. Data were expressed as a percentage of TUNEL-positive nuclei to the total number of nuclei evaluated per section. Quantitative analysis was performed on six heart sections from each rat and five randomly selected fields per section at ×200 magnification were analyzed.

2.13 C-kit, Ki-67 and cardiac troponin T stainings

C-kit$^+$ progenitor cells were identified in the heart by immunofluorescence using a c-Kit antibody on paraffin sections. Briefly, fixed myocardial sections were deparaffinized, rehydrated and microwaved with 10 mM citrate buffer (pH 6) for 30 min followed by permeabilization in 1% Triton X-100 for 20 min. Sections were blocked with normal saline solution supplemented with 0.025% Tween 20 and 2% nonfat milk and incubated overnight with c-Kit primary antibody (diluted 1:200). Next, sections were washed with PBS and then incubated with Alexa 647-conjugated anti-rabbit secondary antibody (diluted 1:500) for 1 h and then mounted in PBS-glycerol with DAPI. Double immunostaining was also performed in paraffin heart sections with Ki-67 (diluted 1:100) and cardiac troponin T (cTnT, diluted 1:200) antibodies. Antigen retrieval was performed using 10 mM citrate buffer (pH 6) for 30 min without permeabilization step. Following overnight incubation with the two primary antibodies, sections were washed with TBS (pH 7.4) and incubated with Alexa secondary antibodies (diluted 1:200) for 1 h and then mounted in PBS-glycerol with DAPI.

2.14 Statistics

Results are expressed as mean ± SEM. Statistics was calculated with Prism 5.0 software (Graphpad Software Inc., San Diego, CA, USA). Changes in variables from baseline to 90 days after treatment were analyzed with the paired *t* test or Wilcoxon signed rank test when values followed a parametric or non-parametric distribution, respectively, within each group. The differences among the groups were first evaluated using the Kruskal–Wallis Test, followed by Mann–Whitney U-test when values followed a non-parametric distribution. The differences among the groups were assessed by ANOVA with a Tukey post hoc correction when the measured values were normally distributed. Shapiro–Wilk test was used to justify the use of a parametric test. A value of $P<0.05$ was considered statistically significant.

3. Results

3.1 FGF-1 and NRG-1 induce adult cardiomyocyte proliferation in vitro

The ability of FGF-1 and/or NRG-1 to stimulate a proliferative response of adult cardiomyocytes was initially studied *in vitro*, in the murine HL-1 cardiomyocyte cell line. Cardiac cells were subsequently treated with the free cytokine for 3 days, revealing a 1.6–2.1 fold increase in cell proliferation when treated with FGF-1 at any dose (Fig. 1). These increases were similar to that observed when HL-1 cells were treated with NRG-1 (1.7–1.9 fold) at the same concentrations. Conversely, FGF-1 and NRG-1 combined treatment also induced a significant increase (1.4-1.6 fold) in the cell proliferation activity similar to the effect induced by the cytokines alone (P=NS).

3.2 FGF-1/NRG-1 combined treatment decreases cardiomyocyte apoptosis in vitro

The protective effects of FGF-1 and NRG-1 were also assessed in HL-1 cells when maintained in serum-free media and at 0.5% O_2 for 2 days. Hypoxia and serum deprivation were sufficient to induce apoptotic cell death, with a 3.1-fold apoptosis increase ($P<0.001$) compared to standard conditions (normoxia and 10% serum). Addition to the cardiac cells of FGF-1 or NRG-1 alone did not exert a significant protective effect but FGF-1/NRG-1 combined treatment, significantly reduced apoptosis induction (measured as DNA fragmentation) when treated at 10 ng/ml (33.5 ± 15.2% decrease) and 100 ng/ml (42.6 ± 12.6% decrease) (Fig. 2A). These results were corroborated by detection of caspase-3/7 activity where a significant decrease was detected when cells were treated with both cytokines at 100 ng/ml concentration (Fig. 2B). These findings demonstrate a synergistic effect of FGF-1 and NRG-1 to protect HL-1 adult cardiomyocytes against apoptosis.

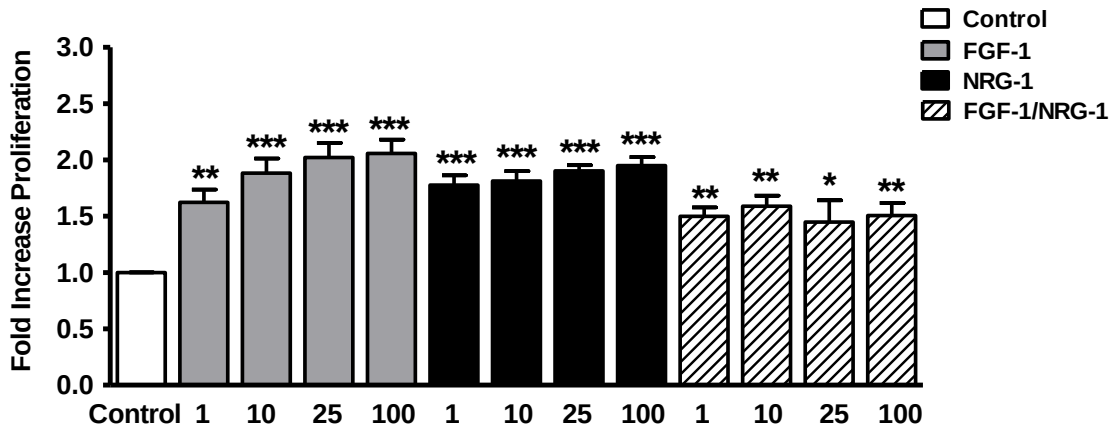

Fig. 1. FGF-1 and NRG-1 induce cardiomyocyte proliferation *in vitro*. Murine HL-1 cardiomyocyte-cell line was treated either without (control) or with the indicated concentrations of FGF-1 and/or NRG-1 (1, 10, 25 and 100 ng/ml) and proliferation rate determined after 3 days; *y* axis represents fold increase *versus* control group. Data are expressed as mean ± SEM from three independent experiments. *$P<0.05$, **$P<0.01$ and ***$P<0.001$ *vs*. control.

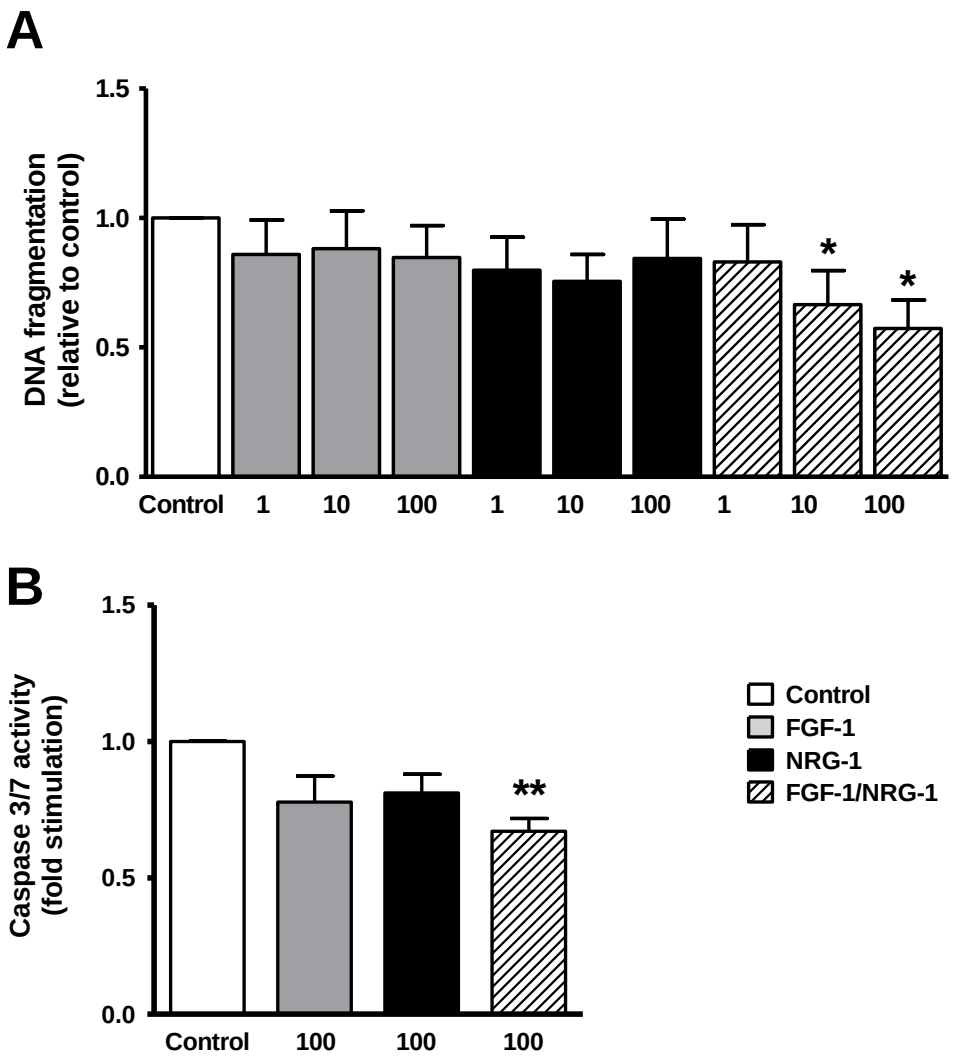

Fig. 2. Effects of FGF-1/NRG-1 treatment on apoptotic cell death of HL-1 cardiomyocytes. HL-1 cells were maintained in serum-free Claycomb medium at 0.5% O_2 for 2 days and either left untreated (control) or treated with the indicated concentrations of FGF-1 and/or NRG-1 (1, 10 and 100 ng/ml). Apoptosis was quantified with two independent assays: **A**. ELISA detection of Histone-associated DNA fragmentation (Data are expressed as mean ± SEM from four independent experiments) or **B**. Detection of Caspase-3/7 activity (Data are given as mean ± SEM for three independent experiments); *y* axis represents fold stimulation *versus* control group. **P*<0.05, ***P*<0.01 *vs.* control.

3.3 Characterization of PLGA microparticles, growth factor release and bioactivity

PLGA microparticles were produced by TROMS with a compatible size for heart injection of 5 µm [18]. Morphological appearance of the factor-loaded microparticles was examined by SEM, revealing a spherical shape with a smooth

surface and few small pores in some particles (Fig. 3A). There were no differences on morphology between FGF-1 and NRG-1 microparticles.

Concerning to the entrapment efficiency, growth factors were efficiently encapsulated, reaching values of 87.4 ± 2.3% for FGF-1 and 65.5 ± 5.1% for NRG-1, that corresponded to final loadings of 874.1 ± 23.4 ng of FGF-1 and 655.3 ± 50.1 ng of NRG-1 per mg of polymer.

Regarding to the *in vitro* release profiles of the growth factors, an initial faster release was observed for the NRG-1-MP, although, after the initial burst, both factors displayed very similar release rates from day 7 to day 28, with 65% of NRG-1 and almost 70% of FGF-1 being released within 28 days (Fig. 3B).

The bioactivity of the released cytokines was evaluated *in vitro* by determining the induction of HL-1 cardiomyocytes proliferation. As shown in Fig. 4, MTS assay revealed a 1.6 and 1.5-fold increase in cell density when stimulated with FGF-1 and NRG-1, respectively. Proliferation rates of cells treated with the free cytokines were practically identical to the ones released by microparticles (1.7-fold for free FGF-1 and 1.5-fold for free NRG-1), indicating that both cytokines retained its biological activity after encapsulation into PLGA-MP.

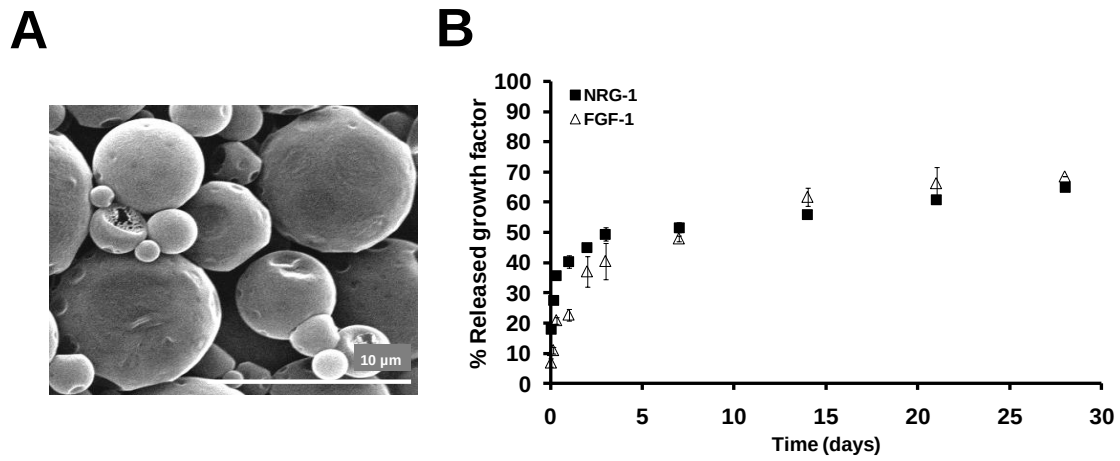

Fig. 3. PLGA microparticles characterization. A. Scanning electron micrograph of PLGA microparticles loaded with FGF-1. **B**. *In vitro* release of FGF-1 and NRG-1 from PLGA microparticles.

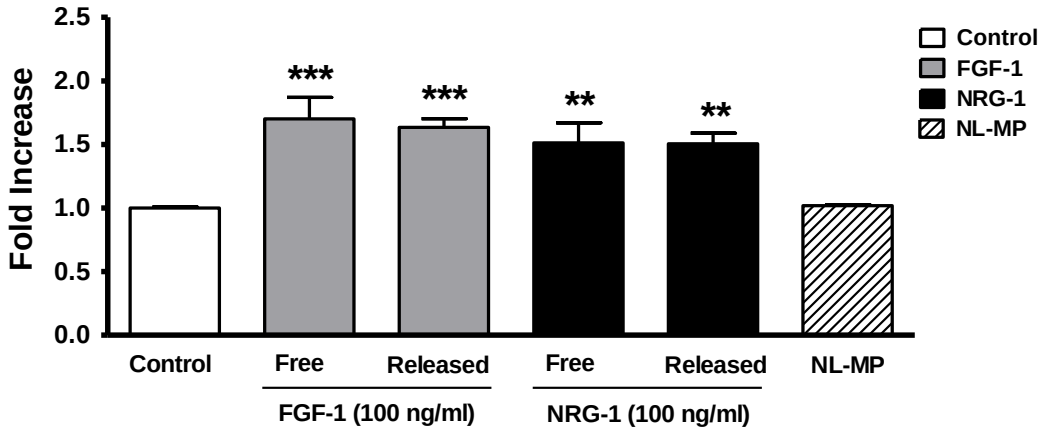

Fig. 4. *In vitro* bioactivity of FGF-1 and NRG-1 cytokines released from PLGA microparticles. Proliferation of HL-1 cells was induced by free or MP-released growth factors at a concentration of 100 ng/ml. A similar proliferation rate was detected after 3 days treatment. Non-loaded microparticles (NL-MP) did not provide a proliferative stimulus nor a toxic effect on the HL-1 cells; *y* axis represents fold increase *versus* control group. Data are expressed as mean ± SEM from three independent experiments. $^{**}P<0.01$ and $^{***}P<0.001$.

3.4 Temporal retention of PLGA microparticles in the heart tissue

The capacity of PLGA microparticles to remain in the tissue for a prolonged period of time is a mandatory requirement for sustained growth factor treatment. In

order to assess the retention of the microparticles in the myocardium, rhodamine-labeled microparticles were injected into the peri-infarct area of the hearts and presence of the particles analyzed after 30, 60 and 90 days of injection. The fluorescent-labeled microparticles were visible by fluorescence microscopy for up to 90 days post-implantation (Fig.5), indicating their long-term presence into the heart.

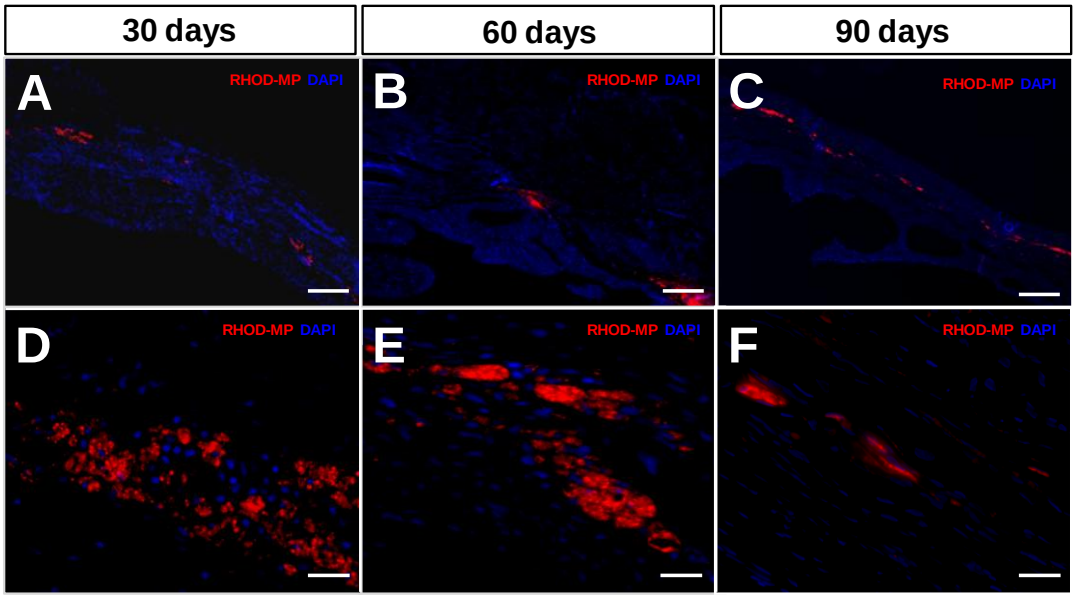

Fig. 5. Temporal retention of PLGA microparticles in the ischemic rat heart. Tissue distribution of rhodamine labeled-microparticles (factor-free) 30 (**A, D**), 60 (**B, E**) and 90 days (**C, F**) after intramyocardial injection. Nuclear staining was performed with DAPI (blue). Scale bars: 100 µm (A-C) and 20 µm (D-F).

3.5 Cardiac Function

Measurements of left ventricular ejection fraction (LVEF) showed that the cardiac dysfunction was maintained after 90 days in rats that received NL-MP (ΔLVEF: 1.1 ± 3.6%). In contrast, absolute changes in LVEF were significantly greater in the rats treated with FGF1-MP (16.7 ± 4.9%, $P<0.05$), NRG1-MP (18.0 ± 5.7%, $P<0.05$) or FGF1/NRG1-MP (13.0 ± 1.9%, $P<0.05$) when compared with the NL-MP group (1.1 ±

3.6%) and similar among the three growth factor-loaded MP treatments (*P*=NS) (Fig. 6).

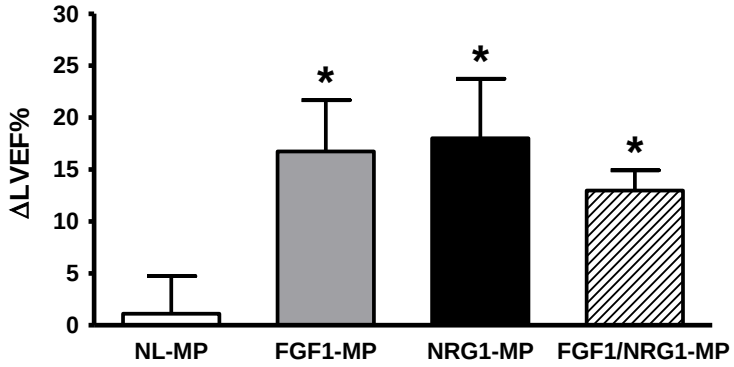

Fig. 6. Growth factor-loaded MP therapy results in improved cardiac function. Left ventricular ejection fraction (LVEF) was measured by echocardiography and expressed as an absolute difference between pre- and post-treatment measurements (3 months post-infarction LVEF – baseline infarction LVEF). Values: mean ± SEM; **P*<0.05 *vs.* NL-MP control group.

Moreover, left ventricular end-systolic and end-diastolic diameters and volumes significantly increased in the NL-MP control group, consistent with a LV chamber dilatation and progression of myocardial dysfunction. In contrast, very significant improvements on heart function could be observed in the NRG1-MP group for LVEDV, as well as LVESV (*P*<0.01 vs. NL-MP, Table 1). Yet, rats treated with NRG1-MP or FGF1/NRG1-MP exhibited a remarkably reduced LVEDV at 90 days follow-up. Finally, the effect of FGF-1 and/or NRG-1 delivery on LV mass was also assessed. No significant differences were observed between baseline and 90 days LV mass within each growth factor-MP group in contrast with the NL-MP group that significantly increased at 90 days (not shown). Table 1 also shows that changes on LV mass normalized for body weight were significantly reduced in all growth factor MP-treated rats, unlike NL-MP group that presented dilation of the ventricle as consequence of a deleterious heart remodeling.

3.6 Morphometric characterization

Histological analysis revealed a reduction in infarct size 3 months after growth factor-MP treatments (alone or in combination) in comparison with the NL-MP control group (NL-MP: 16.8 ± 2.8%; FGF1-MP: 11.9 ± 3.8%, $P<0.01$; NRG1-MP: 12.3 ± 3.6%, $P<0.01$; FGF1/NRG1-MP: 11.7 ± 3.8%, $P<0.01$) (Fig. 7). No difference was found among FGF1-MP, NRG1-MP and FGF1/NRG1-MP therapy. Also, a positive effect was detected when tissue fibrosis was analyzed. A significant lower collagen deposition was detected in the infarcted hearts treated with FGF1-MP (48.7 ± 4.9%, $P<0.001$), NRG1-MP (49.3 ± 2.9%, $P<0.01$) or FGF1/NRG1-MP (44.7 ± 1.3%, $P<0.001$) when compared with the NL-MP group (67.2 ± 1.8%) (Fig. 8). Again, no significant differences were observed among the three groups. Finally, a positive remodeling was detected after treatment with cytokine-loaded MPs. A significantly greater thickness of the LV wall was detected in the FGF1-MP (2.06 ± 0.18 mm, $P<0.05$) and FGF1/NRG1-MP (1.93 ± 0.14 mm, $P<0.05$) treated animals in comparison with the control group (NL-MP: 1.55 ± 0.14 mm). Also, a borderline significant increase in the LV wall thickness was found in the hearts injected with NRG1-MP (1.67 ± 0.07 mm, $P=0.055$ *vs.* NL-MP group).

Table 1. Cardiac function data by echocardiography

	NL-MP	FGF1-MP	NRG1-MP	FGF1/NRG1-MP
LV mass	0.19±0.37	−1.44±0.46*	−2.23±0.67**	−1.38±0.41*
LVEDV	1.50±0.80	−1.61±0.80*	−3.36±0.90**	−2.22±0.70**
LVESV	0.75±0.70	−1.62±0.60*	−2.87±0.70**	−1.90±0.40*
LVEDD	−0.17±0.20	−0.84±0.20$^{\#}$	−1.27±0.30*	−0.96±0.20*
LVESD	−0.22±0.20	−0.93±0.20*	−1.25±0.20**	−0.99±0.10*
E/A ratio	−0.10±0.07	−0.10±0.09	−0.19±0.11	−0.03±0.08

LV mass, volumes and diameters were normalized for body weight. Values (mean ± SEM) expressed as an absolute difference between pre- and post-treatment measurements (3 months post-infarction – baseline infarction). LV mass (mg/g); LVEDV: left ventricular end-diastolic volume (ml/Kg); LVESV: left ventricular end-systolic volume (ml/Kg); LVEDD: left ventricular end-diastolic diameter (cm/Kg); LVESD: left ventricular end-systolic diameter (cm/Kg); E/A: peak E and A transmitral filling velocity ratio. $^{\#}P$=0.07, *P<0.05, **P<0.01, vs. NL-MP control group.

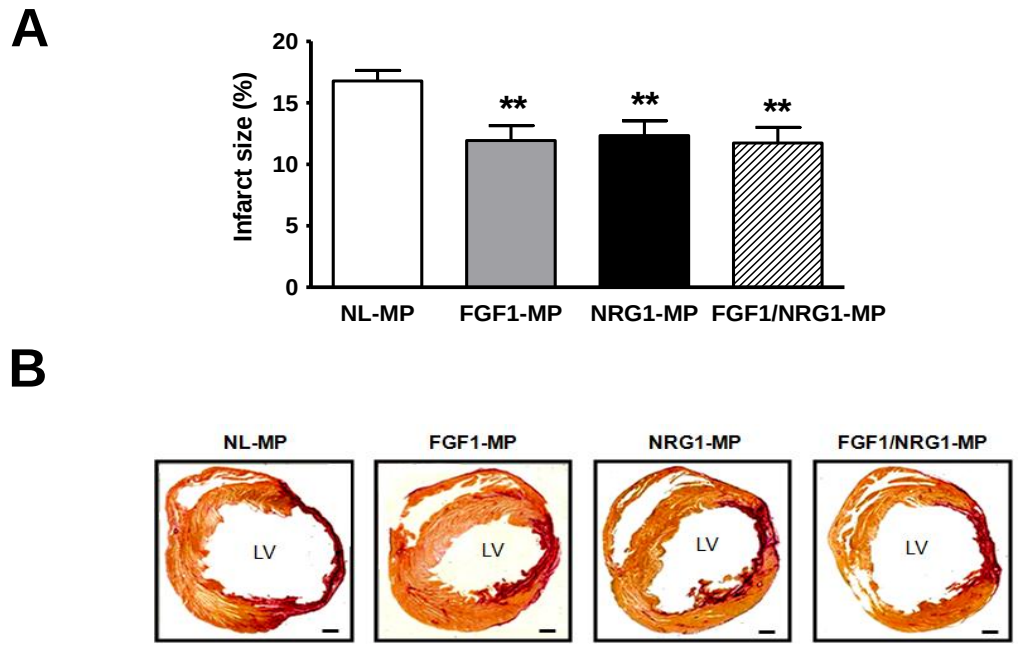

Fig. 7. Infarct size. A. Measurement of heart infarct size: Mean ± SEM, ***P*<0.01 *vs.* NL-MP control group. **B**. Representative images of Sirius red stainning of infarcted left ventricle from each treatment group. Scale bars: 1 mm.

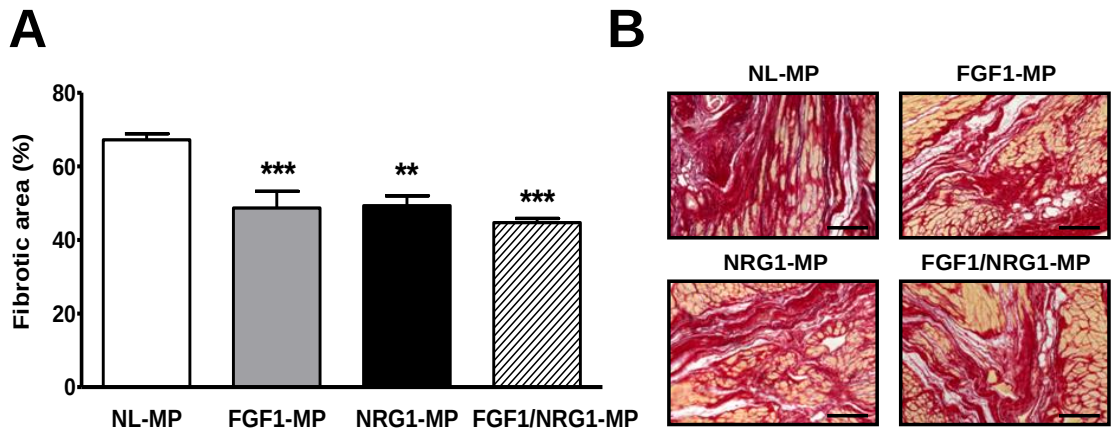

Fig. 8. Tissue fibrosis A. Fibrosis degree in the infarcted zone. Mean ± SEM, ***P*<0.01 and ****P*<0.001 vs. NL-MP control group. **B**. Representative Sirius red images. Scale bar: 200 µm.

3.7 Vascular density

Quantification of SMA^+ vessels indicated a significant greater revascularization of the hearts treated with FGF-1 and/or NRG-1 microparticles than hearts treated with NL-MP (Fig. 9A). The arteriolar/arteries densities in the injured zones after 90 days of

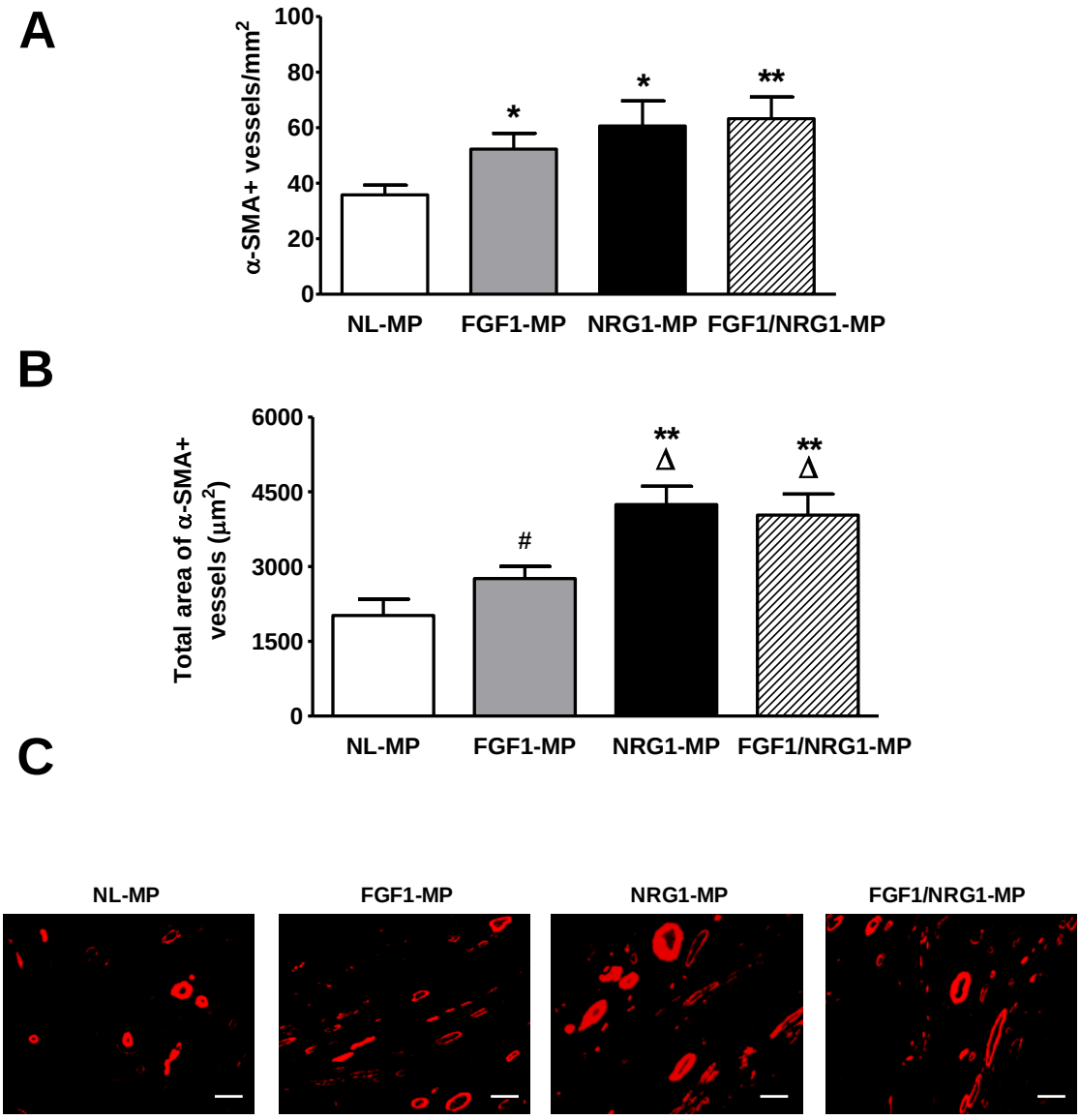

Fig. 9. Arteriogenic effects of FGF-1 and NRG-1 released from PLGA-MP in the ischemic myocardium A. Arteriolar density. Mean ± SEM, **P*<0.05 and ***P*<0.01 *vs*. NL-MP control group. **B**. Quantification results of the total area occupied by α-SMA-positive-vessels (μm^2). Mean ± SEM, $^{\#}$*P*=0.08 *vs*. NL-MP control group; ***P*<0.01 *vs*. NL-MP control group; $^{\Delta}$*P*<0.05 *vs*. FGF1-MP group. **C.** Representative images for α-SMA$^+$ immunofluorescence stainnings. Scale bar: 50 μm.

injection were as follows: 36 ± 5 α-SMA$^+$ vessels /mm^2 in the NL-MP group; 52 ± 7 α-SMA$^+$ vessels /mm^2 in the FGF1-MP group (*P*<0.05); 60 ± 10 α-SMA$^+$ vessels /mm^2 in the NRG1-MP group (*P*<0.05); and 63 ± 8 α-SMA$^+$ vessels /mm^2 in the FGF1/NRG1-MP group (*P*<0.01) *vs*. NL-MP group (Fig. 9A). Also, the α-SMA$^+$-

vessels area was determined, showing a significant increase after administration of any of the microparticle treatments. Interestingly, NRG-1 treatment (alone or combined with FGF-1) was more efficacious than the FGF-1 one (FGF1-MP: 2759 ± 243 μm^2; NRG1-MP: 4245 ± 366 μm^2, $P<0.05$; FGF1/NRG1-MP: 4032 ± 422 μm^2, $P<0.05$ vs. FGF1-MP) (Fig. 9B).

3.8 Myocardial apoptosis

Myocardial apoptosis was analyzed by TUNEL staining. Similar levels of cell death were detected in the hearts treated with the NL-MP or the cytokine-MP alone (NL-MP: 2.8 ± 0.9%; FGF1-MP: 1.5 ± 0.2%, P=NS; NRG1-MP: 1.8 ± 0.6%, P=NS). However, a clear trend in tissue protection was detected when cytokine combination was used (NL-MP: 2.8 ± 0.9% *vs.* FGF1/NRG1-MP: 1.1 ± 0.3%; P=0.08), showing, as it was observed in the *in vitro* experiments, a synergistic protective effect of FGF-1 and NRG-1.

3.9 Recruitment of c-Kit+ cells

Antibody staining of myocardium revealed that c-Kit^+ cells could be found near or inside to the blood vessels in the infarcted area of FGF1/NRG1-MP-treated hearts (Fig. 10). Importantly, more c-Kit^+ cells were detected in the FGF1/NRG1-MP group than NL-MP control group. This population was identified as isolated or clustered cells that were probably recruited towards the ischemic myocardium. Importantly, some $cTnT^+$ cells were also Ki-67^+, which indicates cardiomyocyte proliferation. Of note, we found small spherical $cTnT^+$/Ki-67^+ cells, which we speculated could be newly, formed cardiomyocytes.

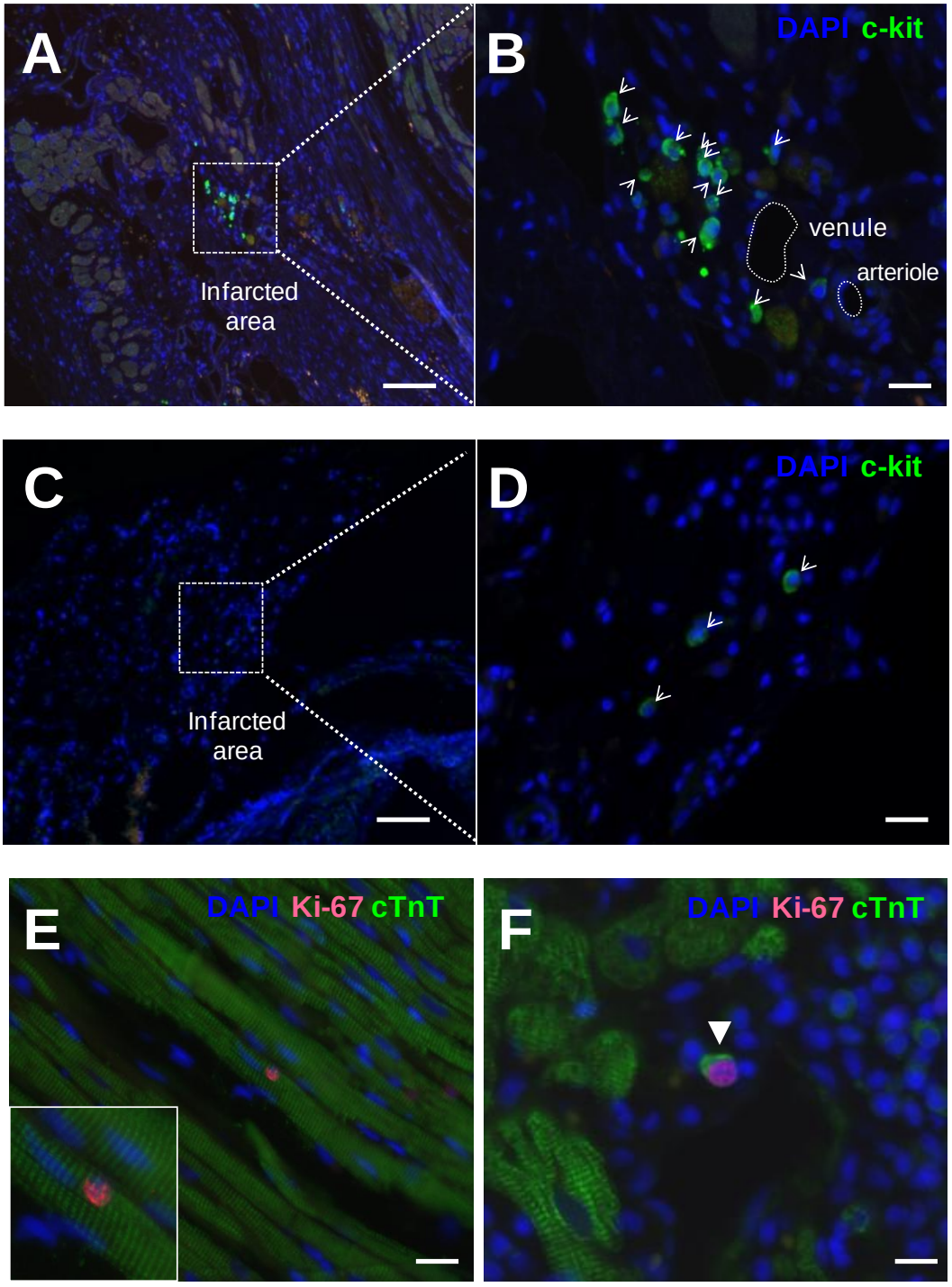

Fig. 10. Immunofluorescence for c-Kit, Ki-67 and cardiac troponin T on heart sections. A-B. c-Kit$^+$ cells were detected in the surroundings or the lumen of the vessels along the infarcted area of rats treated with FGF1/NRG1-MP, which suggests recruitment of c-Kit$^+$ progenitor cells towards that zone. Importantly, more c-Kit$^+$ cells were detected in the FGF1/NRG1-MP group than NL-MP control group **(C-D). E**. Representative image of a proliferating Ki-67$^+$ adult cardiomyocyte (pink nucleus) in the FGF1/NRG1-MP group. Myocytes were stained by a cardiac troponin T (cTnT) antibody (green). Nuclei were stained by DAPI (blue). **F.** Representative image of a cTnT$^+$/Ki-67$^+$ cell (arrowhead) in the FGF1/NRG1-MP group, suggesting a newly formed cardiomyocyte. Scale bars: 100 µm (A and C) and 20 µm (B, D, E and F).

4. Discussion

Cardiomyocyte proliferation and survival are two key mechanisms to be targeted in order to promote cardiac regeneration. FGF-1 and NRG-1 proliferative and protective effects have been confirmed in our study as it was previously reported. Effectively, we have observed a proliferative induction in a cardiomyocyte cell line (HL-1) when FGF-1 or NRG-1 were added to the cell culture. Also, a putative synergistic effect of both cytokines together was assessed although not greater effects were detected. Previous studies reported this lack of synergia too [21] probably due to the activation of a common cell signaling for cardiomyocyte cell-cycle reentry [20, 22].

On the contrary, a synergic effect was detected when cardiomyocyte survival was assessed in the presence of both cytokines. It is worth noting that single factors exhibited poor or negligible anti-apoptotic effects in our studies. These were unexpected results because both FGF-1 and NRG-1 have been reported to separately decrease cardiomyocyte apoptosis [25, 29]. Cardioprotective FGF-1 effects have been widely studied in models of hypoxia and reoxygenation [28, 29]. In turn, NRG-1 has been shown to protect cardiomyocytes against apoptosis in response to chemotherapeutic agents [33]. These different results might be due to methodological differences, such as the use of a cardiomyocyte cell line instead of primary cultures among other aspects [25, 34, 35].

In view of the effects of FGF-1 and NRG-1 upon proliferation and survival of cardiomyocytes, we tested whether this could translate into a therapeutic benefit after heart MI. For that, we used biocompatible and biodegradable PLGA microparticles to deliver FGF-1 and NRG-1 in the myocardium in a continuous and controlled manner. We have demonstrated that PLGA microparticles remain in the heart tissue for up to 90

days, favoring a long-term growth factor treatment. FGF-1 and NRG-1 were successfully encapsulated into PLGA microparticles, which released the factors in a controlled fashion for up to 28 days *in vitro*. Importantly, the released growth factors were biologically active, as demonstrated by the enhanced proliferation of HL-1 cells *in vitro*, indicating that the microencapsulation process does not alter their bioactivity.

A number of approaches have been designed to deliver growth factors in the heart in a controlled fashion. These include hydrogels, peptide nanofibers, liposomes, nano- and microparticles mainly for delivery of VEGF [18, 36-39], FGF-1[30] and FGF-2 [40, 41]. Although hydrogels are an appealing class of delivery vehicle, it has been reported some technical difficulties to inject gelatine hydrogel into the thin ventricular wall of the infarcted rat heart [42, 43]. Some reports have also demonstrated the accumulation of liposomes in the areas of experimental myocardial infarction [37, 44, 45], but the clinical application of liposomes is still hindered due to their instability and their interaction with high-density lipoproteins in blood. Here, we report a long-term preservation of the heart function after FGF-1 and/or NRG-1 microparticle treatment.

From echocardiographic data, administration of NRG1-MP combined or not with FGF1-MP, significantly reduced the severity of LV hypertrophy, with attenuated LV systolic dimensions. In contrast, LV volumes and dimensions were similar at baseline and 90 days post-treatment in the FGF-1 MP treated hearts. It has been reported the FGF-1 hypertrophic action [46, 47] and, therefore, it could be reflected on LV dimensions. Still, a positive effect of FGF1-MP can be reported as avoided heart dilation as occurred in the control hearts treated with the NL-MP. Moreover, improved cardiac function was also associated with an attenuation of ischemic injury at the tissue

level. All three growth factor-loaded MP treatments preserved the myocardium from fibrosis and reduced the infarct size and mass lost of the LV wall, which displayed significantly thicker compared to those in rats injected with NL-MP. Importantly, previous studies where NRG-1 was intravenously freely injected, did not found differences in infarct size in comparison with the control group [48]. Although this parameter was analyzed at a much earlier time-point, it is possible that the more robust effect observed in our study might be due to the continuous and stable release of the cytokine by the microparticles.

We sought to explore the underlying mechanisms that have controlled the cardiac repair and led to functional improvement of infarcted rats. Tissue revascularization was assessed in all the animal groups, and a significantly increased arteriolar/arteries density was found in all the growth factor-loaded MP groups, which probably could improve collateral flow and LV function. Interestingly, when the SMA^+-vessels area was measured, only those groups treated with NRG-1 presented a significant increase, showing a greater revascularizative effect of the NRG-1 cytokine in comparison with FGF-1.

Tissue apoptosis was also assessed, as it has been shown to be a key factor in the development and progression of post-MI remodeling [49, 50]. TUNEL staining showed a clear trend in the anti-apoptotic effect exerted by the combined treatment of FGF1 and NRG1-MP treatment at 90 days follow-up. It would be interesting in order to confirm this effect to analyze the protective effect of the particles at earlier stages of healing and remodeling when probably a more potent was being exerted by the cytokine treatment [51].

On the other hand, animal studies of myocardial regeneration suggested salutary effects of stem cell transplantation, mainly by secretion of trophic factors involved in cell survival and progenitors recruitment, differentiation and proliferation [52, 53]. It has been reported that bone marrow-derived c-Kit$^+$ cells are required for the endogenous cardiac repair process after MI [54]. Engel *et al.* have speculated that FGF1/p38 MAP kinase inhibitor therapy might increase proliferation of resident stem cells in the heart or peripheral stem cells recruited to the heart [30] and it is also possible that NRG-1 can induce undifferentiated cardiac progenitor cells to proliferate and differentiate into adult cardiomyocytes [55, 56]. Here, we have identified c-Kit$^+$ cells localized near to the blood vessels lumen or in their lumen at the infarcted area of the hearts treated with FGF1/NRG1-MP. It is formally possible that these progenitors might have been recruited towards the ischemic myocardium under stimulation of the released FGF-1 and NRG-1. In view of the enhanced arteriolar/arteries density and the location of some c-Kit$^+$ cells into the arterioles, it can be hypothesized that enhanced arteriogenesis could have been mediated by myocardial recruitment and subsequent differentiation of c-Kit$^+$ progenitor cells into a smooth muscle phenotype, as it has been previously described [57]. Their cardiac capacity of the c-Kit$^+$ cells has been also described. It will be interesting to identify in more detail the origin of this cell population. On the other hand, proliferating Ki-67$^+$ adult cardiomyocytes were detected in the hearts treated with FGF1/NRG1-MP, which could indicate cardiomyocyte replacement after cytokine stimulation. Interestingly, Ki-67$^+$ small cTnT$^+$ cells were also found, presenting a small cytoplasm and lack of sarcomeric bands. We hypothesized that these cells could be identified as proliferating cardiac progenitor cells completing their differentiation into mature cardiomyocytes.

Taken together, our present findings strongly suggest the therapeutic benefit of growth factors when combined with protein delivery systems for cardiac regeneration. They provide a successfully example of single or combinatorial growth factor delivery by using a slow-release polymer, which shown to be effective for carrying FGF-1 and NRG-1 in the ischemic myocardium. As result, this strategy positively affects post-MI remodeling, which significantly contributed to global myocardial function.

5. Conclusions

Our results show that single or combinatorial therapy with FGF-1 and NRG-1 delivered by PLGA microparticles improved ventricular function, by attenuating deleterious heart remodeling, promoting arteriogenesis and cardiac proliferation, and recruiting cardiovascular progenitor cells. This strategy of local and controlled delivery of FGF-1 and NRG-1 may prove useful for treating patients with myocardial injury and therefore warrants investigation on pig pre-clinical myocardial infarction model.

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GENERAL DISCUSSION

Cardiovascular diseases (CVD) are the leading global cause of death in both men and women, extending across all racial and ethnic groups [1]. In the United States (US) population, CVD total mention deaths (1.36 million in 2004), accounted for about 57% of all deaths [2]. In Europe, these pathologies account for over 4.30 million deaths each year, which represents nearly half (48%) of all deaths in this continent [3]. CVD are also the leading cause of death (31.2% of all deaths in 2009) and hospitalization in Spain, being associated with around 125000 deaths and 5 million hospital admissions each year [4]. A large number of asymptomatic individuals are at a high risk of CVD because they have two or more risk factors. In more than 60% of these individuals, risk factors are not dealt with properly. Moreover, the situation is improving only slowly in Spain, according to data provided by *Instituto Nacional de Estadística (INE)*. More than a third of patients who have had an acute myocardial infarction die before reaching hospital without receiving appropriate treatment. For these patients, prevention is too late. As a result, CVD incidence and mortality in Spain have not improved substantially in the past decade. Ischemic heart disease (IHD) is the main problem within CVD, including coronary heart disease (CHD) and stroke. Just under half of all deaths from CVD are from CHD and nearly a third are from stroke [3]. IHD results from the blockage in coronary arteries by atherosclerosis and thrombus and presents with symptoms such as temporary pain (angina), irregular heart beat (arrhythmia), permanent heart muscle damage (myocardial infarction) and loss of muscle activity (heart failure).

Endothelial dysfunction has been implicated in the pathogenesis of many diseases affecting the cardiovascular system. Experimental and clinical studies have shown that endothelial dysfunction may play a key role in diverse conditions such as abnormal arterial vasomotion, thrombosis, and neointimal proliferation [5]. Endothelial

dysfunction is a characteristic of atherosclerotic vessels, arteries subjected to mechanical injury, and collateral vessels that develop in response to severe ischemia. When a major artery becomes obstructed, blood flow to the ischemic tissue often depends on collateral vessels. When spontaneous development of collateral vessels is insufficient to allow normal perfusion of the tissue at risk, residual ischemia occurs. A growing body of evidence indicates that abnormal vascular reactivity may limit the beneficial effects of collateral vessels on tissue perfusion. It has been demonstrated that this abnormal reactivity occurs, at least in part, as a consequence of dysfunctional endothelium [6].

Each day, patients are admitted to hospital with chest pain due to coronary artery occlusion and insufficient oxygen delivery to cardiomyocytes [7]. Conventional medical treatments include drugs, mechanical revascularization techniques and surgery (Table 1). For a number of patients, the only alternative is organ transplantation. Anti-thrombotic drugs suppress the formation of thrombin, preventing the formation of blood clots. These drugs aim to prevent development and progression of thrombosis, promote dissolution or stabilization of acute and residual mural thrombus, and reduce thromboembolism, myocardial infarction and death. Unfractionated heparin, Factor Xa inhibitor (fondaparinux) and direct thrombin inhibitor (DTI) are the most important anti-thrombotic drugs for patients with acute coronary syndrome (ACS) such as unstable angina (UA) and non-ST-elevation myocardial infarction (NSTEMI) [8, 9]. Other pharmacological treatments include angiotensin-converting enzyme (ACE) inhibitors and β-blockers in combination with diuretics. These drugs can reduce preload and afterload to the heart to a varying extent, block the trophic effects of the activated reninangiotensin-system and reduce arrhythmogenicity [10]. Adenosine, antioxidants,

anti-platelet agents, thienopyridines and cholesterol-lowering medications are also frequently used. Regarding revascularization strategies, percutaneous coronary intervention (PCI) is a medical procedure based on the inflation of a balloon within the blocked coronary artery to destroy the plaque into the walls of the artery and recover normal blood flow to the myocardium. A drug eluting stent can be optionally introduced into the blood vessel or artery. In fact, stenting is an alternative to heart surgery for some forms of non-severe coronary artery disease. This procedure is effective in mostly acute heart attack and reduces mortality from coronary artery disease compared with a standard treatment regimen through administering anti-thrombotic drugs [11]. Surgical procedures like coronary artery bypass grafting (CABG) are also frequently performed to reduce the risk of death from coronary artery disease.

Although the use of advanced revascularization strategies such as PCI or CABG in patients with ischemic heart disease has resulted in a marked decrease in mortality, a significant number of these patients are not candidates for coronary revascularization procedures or achieve incomplete revascularization with these procedures. About one out of five patients cannot be treated with, or does not profit adequately from these current standard therapies [12]. Consequently, many of these patients have persistent symptoms of myocardial ischemia despite intensive medical therapy. They may suffer from severe diffuse atherosclerotic disease not amenable to surgery or angioplasty or they may have had prior revascularization procedures, hampering future repeat procedures. These patients continue to live with symptomatic obstructive vascular disease resulting in lifestyle-limiting claudication and limb ischemia, intractable angina, and congestive heart failure. Improving the quality of life, morbidity, and mortality of

this growing patient population is a major challenge facing the future of cardiovascular medicine.

Even the best conventional treatment regimens will not cure the patients. The myocardium will remain damaged since the mammalian heart is not able to significantly regenerate itself. Conventional therapy may, at best, slow the progress of heart failure. This clinical situation has led to extensive investigation to find new treatments. Among others, researchers and clinicians have focused on restoring blood flow by inducing neovascularization by treatment with cells, genes and growth factors involved in this process.

Table 1. Conventional and modern approaches for CVD treatment

Current standard therapies	**Modern approaches**
Drugs	Cell-based therapies
Reperfusion	Gene therapy
Vascular surgery	Angio- and arteriogenic growth factor therapy
Organ transplantation	

As reviewed in the **Introduction**, angiogenesis involves the extension of the already formed primitive vasculature by the sprouting of new capillaries through migration and proliferation of previously differentiated endothelial cells [13]. In particular, angiogenesis is a natural protective mechanism that provides an endogenous collateral circulation able to protect the downstream tissue from ischemic injury during progressing vascular occlusions. However, while ischemia from vascular occlusion up-regulates expression of angiogenic growth factors, the fact that certain patients present with disabling angina indicates that such natural compensatory processes are not always

sufficient [13, 14]. In fact, in many ischemic diseases, such as peripheral vascular disease, coronary ischemia and chronic wounds, the intrinsic capacity for spontaneous vascular repair and tissue regeneration is severely compromised. Therefore, the stimulation of angiogenesis by growth factors may have therapeutic value for coronary artery disease, cardiac failure and tissue injury, mainly for patients who cannot be adequately treated by conventional treatments such as PCI and CABG [15, 16]. In other words, the goal of therapeutic angiogenesis is to stimulate the tissue revascularization. In the future, this novel therapy could potentially replace surgical revascularization and angioplasty which are more invasive procedures and also prone to restenosis, a complication deriving from scar tissue overgrowth that can renarrow the artery and block blood flow to the heart.

Theoretically, therapeutic angiogenesis can be achieved by employing either growth factor proteins or by introducing genes encoding these proteins [17-19]. Each approach has its advantages and limitations, as summarized in Table 2. Cell therapy-based approaches have also contributed to neovascularization and tissue repair, and will be discussed later. The theoretical advantage of gene therapy approaches with respect to longer-term angiogenic factor exposure depends on effective local expression, which is usually not completely achieved because of high variability in the level and duration of gene expression. On the other hand, prolonged local production of potent growth factors may cause increased vascular permeability and edema or hemangioma formation [7]. Still, gene therapy approaches have additional concerns regarding the introduction of foreign genetic material and exposure to viral vectors [20]. Moreover, while phase I studies obtained promising improvements, multicenter randomized, double blind, and placebo controlled trials showed disappointing results. For example, in the Angiogenic

GENe Therapy (AGENT), an adenovirus vector carrying the FGF-4 gene was delivered by intracoronary route in 79 patients with angina. There was no significant increase in treadmill time compared with placebo at 12 weeks [21]. Other clinical settings such as Euroinject One trial [22], REVASC [23] and RAVE [24] trials also failed to show efficacy of VEGF based on gene therapy. Taken together, these findings suggest that although gene therapy might have great potential to the ischemic heart diseases, nowadays technical limitations such as transfection efficiency and stability and long-term expression of the therapeutic genes must be overcome to be considered in contemporary medical practice.

Table 2. Comparisons between gene and protein therapy

	Gene therapy	**Protein therapy**
Dose	Unpredictable	Predictable
Inflammatory response	Yes	No
Introducing foreign material	Yes	No
Serum half-life	Long	Short
Tissue half-life	Unpredictable	Short, but can be modified
Sustained exposure	Yes	Yes, through controlled released formulations

On the other hand, the major advantage of the protein therapy approach lies in precise knowledge of the delivered dose. This is an important drawback of gene therapy because of the inconsistent level of expression achieved with the same dose in different patients, partly attributable to delivery issues and to variability in the presence and level of neutralizing antibodies [25]. Theoretically, protein therapy does not depend on patient serology and does not consist of injecting foreign material. In fact, experimental

growth factor therapy was spurred on after the development of recombinant production techniques and ways of purifying angiogenic proteins [26-29]. However, the major limitation of the protein therapy approach has been the limited tissue half-life of angiogenic proteins. Inherent protein instability has been another cause of the missing clinical effects. Indeed, clinical trials with FGF-1 [30], FGF-2 [31] and VEGF [32] were disappointing, probably owing to suboptimal protein delivery. Attempts have been made to circumvent the short lived effect and high instability of the protein when injected as a bolus, either by modifying proteins themselves or by protein delivery systems. Regarding the latter, we reviewed approaches for controlled protein delivery dedicated to cardiac regeneration (**Introduction**). These include hydrogels, polymer scaffolds, nano- and microparticles, liposomes, etc... based on synthetic and natural materials. In the context of intensive research in the field of biomaterials and drug delivery platforms applied to cardiac regeneration, polyester-based microparticles (MP) do have the potential for delivering growth factors related to cardiac repair. However, their use in cardiac drug delivery has not been fully investigated.

We sought to explore the potential of poly(lactic–*co*-glycolic acid) (PLGA) MP to deliver growth factors in the myocardium. PLGA has attracted significant interest in drug delivery due to its favorable properties such as good biocompatibility, biodegradability, low immunogenicity and low toxicity [33]. In addition, PLGAs are suitable to formulate into different devices for delivering a variety of drug classes such as vaccines, peptides, proteins, and macromolecules. Also, the US Food and Drug Administration (FDA) have granted the approval of PLGA for human use. As an example, Table 3 lists PLGA-based drug delivery products available on the market.

Table 3. PLGA formulations on the market

Product	Active compound	Formulation	Indication	Company
Arestin®	Minocycline	Microparticles	Periodontal disease	Orapharma
Decapeptyl®	Triptorelin pamoate	Microparticles	Prostate cancer	Ferring
Eligard®	Leuprolide acetate	Implant	Prostate cancer	Sanofi-Synthelabo
Lupron Depot®	Leuprolide acetate	Microparticles	Prostate cancer	TAP
Nutropin Depot®	Growth hormone	Microparticles	Growth failure	Genentech
Profact® Depot	Buserelin acetate	Implant	Prostate cancer	Aventis
Risperidal® Consta™	Risperidone	Microparticles	Antipsychotic	J&J
Sandostatin LAR® Depot	Octreotide acetate	Microparticles	Acromegaly	Novartis
Somatuline® LA	Lanreotide	Microparticles	Acromegaly	Ispen
Suprecur® MP	Buserelin acetate	Microparticles	Prostate cancer	Aventis
Trelstar™ Depot	Triptorelin pamoate	Microparticles	Prostate cancer	Pfizer
Vivitrol®	Naltrexone		Alcohol dependence	Alkernes
Zoladex®	Goserelin acetate	Implant	Prostate cancer	Astrazeneca

Another advantage of PLGA is that controlled drug release can be achieved by adjusting the polymer parameters such as molecular weight and monomer ratio. These advantages have also led to various medical and pharmaceutical applications including sutures, dental repairs, fracture fixation, ligament reconstruction, vascular grafts as well as controlled drug delivery carriers. Also, for neovascularization purposes, PLGA has been used to obtain millicylindrical implants, membranes, scaffolds and nano- and microparticles to encapsulate VEGF [34, 35], FGF-2 [36], PDGF-BB [37] and Ang-1 [38]. However, PLGA-MP have not been thoroughly investigated as a feasible delivery system for growth factors in the myocardium.

The pioneering work developed in this Thesis was designed to investigate thoroughly the cardiac application of PLGA-MP. With that purpose, first of all, we selected an adequate method to encapsulate growth factors into PLGA-MP. A wide range of formulation methods has been used for encapsulating proteins in PLGA-MP. These include solvent extraction, phase separation, spray drying, solid encapsulation, static mixer extrusion, expansion in a supercritical fluid, among others. In order to deliver the desired protein from PLGA-MP for an extended period, two critical issues should be considered: an appropriate protein release kinetic profile and the retention of biological activity after release. To prepare our PLGA-MP, we therefore selected the Total Recirculation One-Machine System (TROMS), a technique based on the multiple emulsion solvent evaporation method. TROMS relies on the turbulent injection of the phases, thus avoiding the use of aggressive homogenization techniques or heating, which makes the method especially useful for the encapsulation of biomolecules [35, 39, 40]. In fact, all three growth factors (VEGF, FGF-1 and NRG-1) that we encapsulated into PLGA-MP maintained their biological activity, which indicates that microencapsulation process by using of TROMS did not alter protein structure as well as bioactivity.

Prior to encapsulating growth factors into PLGA-MP for cardiac delivery, we sought to investigate basic questions related to intramyocardial injection of these particles like determining the most suitable particle size for heart injection, the most appropriate resuspension medium to inject these particles and the long-term permanence of the PLGA-MP in the myocardium and tissue response to them. As presented in **Chapter 1**, TROMS produced PLGA-MP in the size range of 2-30 μm varying apparatus conditions. Particles were negatively charged (–30 mV at pH 7.6) with

minimal content of residual poly(vinyl alcohol) (PVA). Here, it is important to justify the use of PVA as an emulsifier that stabilizes emulsion and prevents MP coagulation during solvent removal [41]. However, as PVA is a potentially toxic non-biodegradable polymer, its administration should be minimized as much as possible. The percentage of PVA recovered in the microparticles ranged from 1.1% to 1.6% for all quantified formulations in this work. These values are several times lower than $13\%_{w/w}$ PVA content previously reported [42].

Next, we assessed the potential of these particles for carrying growth factors in a rat model of MI. We performed a formulation screening based on the particle size for heart injection. Previously, we selected a delivery medium composed of DMEM supplemented with carboxymethylcellulose, polysorbate 80 and mannitol to resuspend and inject the MP in the myocardium. Particles with a diameter of 5 µm were compatible with an intramyocardial administration. They did not induce inflammatory reactions when compared to injection medium alone. Also, they were visible in the heart tissue for up to a month post-implantation, which indicates that MP were not completely degraded at this stage. This is a considerable level of tissue retention, regarding the need for long-term growth factor exposure to the tissue. Seshadri *et al.* tested the retention of a new candidate polymer to deliver therapeutics in the heart, namely, poly(cyclohexane-1,4diyl acetone dimethylene ketal) (PCADK). The results demonstrated retention of the PCADK particles for only 10 days in the myocardium [43, 44]. On the other hand, our PLGA-MP presented a moderate level of macrophage-mediated phagocytosis in the heart tissue. CD68 immunolabeling revealed 31% and 47% microparticle uptake one week and one month after injection, respectively. Developing chemically modified microparticles with a protective PEG layer, which prevent cellular immune reactions,

could circumvent this limitation. This strategy is actually being used in our group [45]. Collectively, the results presented in the **Chapter 1** provided a basis for delivering a growth factor into PLGA-MP and for further therapeutic evaluation in a rat model of myocardial infarction.

The ability of VEGF to promote collateral vessel growth in various animal models of ischemia generated much enthusiasm, but pharmacokinetic and safety issues besides uncontrolled delivery strategies still hinder their success as a pro-angiogenic drug. For therapeutic purposes, the presence of VEGF must not only be localized, but also be sustained to the ischemic tissue area [46]. Regarding these considerations, we prepared PLGA-MP containing $VEGF_{165}$, which is the major VEGF isoform [47] (**Chapter 2**). Encapsulation efficiency values up to 83% were achieved by TROMS. In fact, preparation of VEGF-loaded PLGA-MP using TROMS seemed to be superior to other approaches, where encapsulation efficiency of 67% was achieved when this protein was entrapped into large (~500 μm) alginate beads by using the needle extrusion/external gelation method [48]. Cao *et al.* also reported an entrapment efficiency of 14.5% of VEGF in PLGA-MP, employing the solid-encapsulation method [49]. Kim *et al.* proposed a combination of the multiple-emulsion technique and the atomization-freeze process into a unique solid-encapsulation/single-emulsion/solvent extraction method, but the entrapment efficiency of VEGF into PLGA microparticles using this manufacturing strategy was not improved, achieving a 16% entrapment efficiency [50]. Recently, Golub *et al.* proposed an approach based on PLGA nanoparticles containing VEGF for neovascularization. However, the encapsulation efficiency of VEGF employing a modification of the double emulsion method was only 5.3% [34]. In addition to high encapsulation efficiency, our PLGA-MP provided

sustained release of VEGF, which maintained its bioactivity as demonstrated by its capacity to induce proliferation of HIAECs as well as binding to the KDR receptor.

The next step was to assess the *in vivo* effects of VEGF-loaded MP in a rat model of myocardial infarction induced by ischemia–reperfusion. One month after treatment, an increase in angiogenesis (small caliber caveolin-1 positive vessels) and arteriogenesis (α-SMA-positive vessels) was observed in animals treated with VEGF-MP. The increased revascularization of the tissue translated into a beneficial effect in the remodeling processes, with a significantly greater thickness of the left ventricle wall in the VEGF-MP treated animals in comparison with the control group. These positive effects of VEGF-MP treatment combined with absence of hemangioma, leaking vessels or even tumors indicated that PLGA-MP were able to release bioactive VEGF in a controlled manner, which efficiently activated VEGF receptors in endothelial and, probably, cardiac cells. In summary, our findings presented in **Chapter 2** indicate that the intramyocardial administration of PLGA-MP loaded with VEGF can enhance angiogenic activity in an acute infarct model. Controlled release of VEGF from PLGA-MP significantly increased the vessel density and attenuated ventricular remodeling after MI in a safe manner. These effects were translated in a functional benefit on cardiac function of animals treated with VEGF-MP (not shown).

Next, we sought to explore further mechanisms of cardiac repair that could be therapeutically induced by growth factors. Pre-clinical studies of cardiac regeneration based on stem cell approaches have shown cardiac functional benefits without evidence of actual cell engraftment. A growing body of evidence supports the hypothesis that paracrine mechanisms mediated by factors released by the transplanted cells play an essential role in the reparative processes observed after stem cell mobilization or

injection into the infarcted hearts (reviewed in [51, 52]). Initial experiments showed that injection of conditioned medium obtained from stem cell incubation improved cardiac function and vascular density and reduced infarct size [53]. As the efficacy of cell therapy involves paracrine mechanisms mediated by factors, a wide range of soluble cytokines have been studied to determine the potential for cardiac repair. Also, the ability of these factors to induce mobilization, homing, proliferation, differentiation, angiogenesis, and direct cardiac protection has also been investigated. In addition to VEGF and FGF proteins, examples include neuregulin (NRG), insulin-like growth factor (IGF), stromal-cell derived factor (SDF), hepatocyte growth factor (HGF), platelet-derived growth factor (PDGF), granulocyte/macrophage-colony stimulating factor (GM-CSF), granulocyte-colony stimulating factor (G-CSF), and thymosin β (reviewed in [51]).

Therefore, in the setting of cardiomyocyte death following MI, cardiac repair could be based on recruitment of cardiac progenitors or cardiomyocytes proliferation under growth factor stimulation as it has been reported that differentiated cardiomyocytes may proliferate in response to extracellular mitogens such as periostin [54], FGF-1 [55] and neuregulin-1 (NRG-1) [56]. This evidence supports a new paradigm that the heart is capable of repair and regrowth in response to extracellular mitogens (reviewed in [57]), paving the way for cardiac regeneration by therapeutically using FGF-1 and NRG-1. In addition to the ability to stimulate cell-cycle re-entry and proliferation of differentiated cardiomyocytes, FGF-1 and NRG-1 have been shown to play crucial roles in cardiac repair such as apoptosis mitigation, angiogenesis and recruitment of cardiac progenitor cells and cardiomyocytes maturation. In fact, we confirmed that FGF-1 and NRG-1 promote cardiomyocyte proliferation and survival *in*

vitro, by stimulating a murine cell line of adult cardiomyocytes (HL-1) with FGF-1 and NRG-1 (**Chapter 3**). Despite pro-mitotic and cardioprotective effects, the therapeutic value of FGF-1 and NRG-1 has important limitations *in vivo*, related to their short lived effect and the high instability of protein after systemic administration.

Therefore, we encapsulated FGF-1 and NRG-1 into PLGA-MP and analyzed the beneficial potential of these particles in a rat model of acute MI (**Chapter 3**). We previously assessed the temporal retention of PLGA-MP (factor-free) labelled with rhodamine for a prolonged period of time. The fluorescent-labeled microparticles were visible by fluorescence microscopy for up to 90 days post-implantation, indicating their long-term presence in the heart. Next, FGF-1 and NRG-1 were separately encapsulated into PLGA-MP using TROMS. Again, we prepared 5 µm-sized particles to be adequately injected in the myocardium. Growth factors were efficiently encapsulated, reaching values of entrapment efficiency around 87% for FGF-1 and 65% for NRG-1. FGF-1 has been previously encapsulated into alginate microbeads but very low encapsulation efficiency was reached (around 3%) [58]. In the case of NRG-1, it is the first time that this growth factor has been incorporated into a controlled release system. Thus, the obtained encapsulation efficiencies are a noteworthy result. Regarding the *in vitro* release profiles, both growth factors were sustainably released from the microparticles. An initial faster release was observed for the NRG-1-MP but, after the initial burst, both factors displayed a very similar release rate from day 7 to day 28, with 65% of NRG-1 and almost 70% of FGF-1 being released within 28 days. In the case of FGF1-MP, approximately 23% of the entrapped protein was released within the first 24 hours and around 48% by day 7. After an initial burst in the first few days, FGF-1 release levels remained relatively constant at lower levels for up to four weeks.

Although these data represent an *in vitro* release pattern, they are consistent with the temporal expression of cardiac FGF receptor (FGFR) which increases at day 3, reaches a peak at day 7 and remains high at day 14 after myocardial infarction [59]. Therefore, the release kinetics profile of FGF-1 from the microparticles could potentially follow the FGFR expression timing, which could facilitate the FGF-1/FGFR binding and the triggering of cellular responses related to cardiac repair.

Here, it is important to consider some aspects from MP formulation standpoint for all three growth factors that we have encapsulated (VEGF, FGF-1 and NRG-1). The presence of human serum albumin and PEG 400 as stabilizers protected the growth factor against its denaturing by contact with the organic phase during emulsification. Without stabilizing agents the encapsulated protein acts as a surfactant [60]. It has been reported that PEG reduced the protein anchorage in polymer layers, preventing the release profile to level off even at the late stages of incubation of nerve growth factor (NGF)-loaded PLGA-MP. On the other hand, PEG increased the fraction of protein weakly adsorbed on the polymer surface, leading to a high initial burst effect [61]. Yoncheva *et al.* also reported an initial burst effect during drug release from PLGA-PEG MP, but the PEG modification did not influence the drug loading [62]. Likewise, the selected PLGA (high molecular weight) and PEG 400 could explain the burst effect and the release profile of the growth factors from the PLGA-MP described in the present study. The initial burst could be attributed to diffusion of each growth factor on the surface of the MP, as PLGA degrades forming channels for diffusion. Furthermore, when the protein/polymer ratio is low, the release of proteins from PLGA microspheres is generally regulated by the erosion of the polymer, leading to a faster release at the early stages [63]. However, *in vivo*, a slower growth factor release should be expected.

The main reason is the low availability of water in the tissue compared with the *in vitro* conditions, in which the PLGA-MP are incubated in PBS at 37°C and shaken. Moreover, different proteins which are present in the tissue environment may absorb to the surface of the PLGA-MP and slow the release of the growth factor. Thus, the lack of *in vivo* cytokine release kinetics data is still a limitation for determining the correct cytokine dose. Current studies based on the labeling of growth factors for detecting its release pattern from PLGA-MP in the myocardium are underway in our group. Biotinylation, histidine-tagged or fluorochrome-labeled proteins are being used for that purpose [64, 65]. Other strategy previously reported in the literature is the radiolabeling of growth factors [58, 66].

Regarding the effects of FGF-1 and NRG-1 upon proliferation and survival of cardiomyocytes, we tested whether local and sustained release of these growth factors from PLGA-MP translates into a therapeutic benefit after MI. Three months after treatment, a greater preservation of heart function was observed in rats injected with PLGA-MP containing FGF-1 and/or NRG-1 in comparison with rats that received non-loaded MP (NL-MP, control group). The improvements in cardiac function were accompanied by a positive cardiac remodeling at 90 days follow-up, with a reduced infarct size and fibrosis degree in rats.

Therefore, regarding these positive effects on cardiac remodeling, we sought to explore the mechanisms triggered by FGF-1 and NRG-1 released from PLGA-MP that led to cardiac repair and functional benefit. As vascularization plays a pivotal role on myocardial perfusion and recovery of cardiac function, we quantified the number of α-SMA-coated vessels. An enhanced arteriolar density was found in hearts treated with FGF-1 and/or NRG-1 MP compared to hearts injected with NL-MP. Thus, controlled

released of FGF-1 and/or NRG-1 induces arteriogenesis. Importantly, the presence of NRG-1 (alone or combined with FGF-1) promoted a significant increase in the caliber of SMA^+ vessels. Collectively, these effects upon mature vessels probably could improve collateral flow and LV function.

Regarding the known survival effects of FGF-1 and NRG-1, we also investigated whether apoptosis mitigation could be a further mechanism behind the improvements associated to the treatments with these growth factors. In view of synergistic effects of FGF-1/NRG-1 by decreasing apoptosis of HL-1 cells *in vitro*, we performed TUNEL staining to assess *in vivo* myocardial apoptosis after treatments with the growth factors released from the MP. There were no significant differences on apoptosis level among groups, but a trend towards an anti-apoptotic effect exerted by FGF1/NRG1-MP treatment was detected at 90 days follow-up. As apoptosis occurs at early stages of healing and remodeling after MI [67], it would be interesting to analyze the protective effect of the particles at earlier stages of healing and remodeling when probably a more potent effect was being exerted by the growth factors.

Other possible effects of growth factor treatment could be related to stem cell-based mechanisms, regarding the known trophic activities exerted by soluble factors. In this regard, for example, FGF-1 might increase proliferation of resident stem cells in the heart [68]. In turn, NRG-1 could induce undifferentiated cardiac progenitor cells to proliferate and differentiate into adult cardiomyocytes [69, 70]. Therefore, we sought to investigate the presence of cardiac progenitor cells in the hearts injected with FGF1/NRG1-MP. Immunofluorescence revealed that c-Kit^+ cells localized near the blood vessels or in the lumen of the arterioles. Importantly, more c-Kit^+ cells were semi-quantitatively detected in the FGF1/NRG1-MP group than NL-MP control group, which

could indicate these c-Kit$^+$ progenitor cells probably were recruited towards ischemic myocardium under stimulation of FGF-1 and NRG-1 delivered from the MP. Also, in view of enhanced arteriolar density and the location of some c-Kit$^+$ cells into arterioles, enhanced arteriogenesis could be mediated by myocardial recruitment and the subsequent differentiation of these progenitors into a smooth muscle phenotype, as previously described [71]. Therefore, it is possible that the controlled released of FGF-1 and NRG-1 in the damaged myocardium could induce not only a direct effect upon proliferation of SMA$^+$ cells but also an indirect effect upon cardiac progenitor cells.

Attempts to enhance the therapeutic potential of FGF-1 from a delivery standpoint have employed methods of prolonged growth factor delivery. These include peptide nanofibers [68], alginate microbeads [58] and slow release pump [72]. Regarding the latter, perivascular administration of FGF-1 did not improve blood flow in a pig model of chronic MI [72]. In case of NRG-1, we here report the first approach dedicated to controlled release of this growth factor, which may circumvent its short circulating half-life (approximately 30 min) [73], paving the way for its therapeutic application. Bersell *et al.* demonstrated that NRG-1 improves cardiac function after MI by daily NRG-1 injections later for 12 weeks [56]. Liu *et al.* also reported improved cardiac performance after intravenous injections of NRG-1 for 10 days [73]. These examples of utilization of NRG-1 in the setting of MI indicate its efficacy in promoting cardiac repair in animal models. However, translational potential is difficult because of NRG-1 delivery format (repeated intravascular injections). These regimens are clearly suboptimal, potentially hazard due to systemic exposure and may even be counterproductive. Here, therefore, we report a biocompatible MP formulation to deliver both NRG-1 and FGF-1 in the ischemic myocardium. We demonstrated that this

strategy favorably affected post-MI remodeling, which significantly contributed to global myocardial function.

In summary, PLGA-MP can significantly enhance the efficacy of VEGF, FGF-1 and NRG-1 in the setting of MI. Local and sustained delivery of these growth factors by PLGA-MP can trigger different mechanisms of cardiac repair and consequently promote cardiac regeneration. The use of microparticles could be easily and safely translated to patients if proven in a pre-clinical model.

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GENERAL CONCLUSIONS

The conclusions of the present work are the following:

1. A PLGA microparticle formulation compatible with an intramyocardial injection in terms of particle size, injectability and tissue response was successfully developed by the Total Recirculation One-Machine System (TROMS).

2. $VEGF_{165}$ was efficiently encapsulated into PLGA microparticles. $VEGF_{165}$ remained biologically active after the microencapsulation process, activating its receptor KDR in endothelial cells (HIAEC), and as a consequence of this, promoting the cell proliferation.

3. Tissue revascularization was detected in infarcted rats treated with VEGF-loaded microparticles but not in the animals treated with the free cytokine. Moreover, the newly formed vessels displayed a mature endothelial structure, which indicates safe vascularization induced by the sustained delivery of VEGF from the microparticles. Furthermore, VEGF tissue stimulation translated into a positive remodeling of the infarcted heart.

4. FGF-1 and/or NRG-1 promoted cardiomyocyte proliferation *in vitro*. Also, apoptosis mitigation of adult cardiomyocytes was induced *in vitro* by FGF-1/NRG-1 combinatorial therapy, indicating a synergistic effect of both factors.

5. FGF-1 and NRG-1 were encapsulated into PLGA microparticles using TROMS, yielding high encapsulation efficiencies and showing a sustained in vitro cytokine release. Importantly, the bioactivity of both FGF-1 and NRG-1 was maintained after the microencapsulation process.

6. In a rat model, treatment of acute myocardial infarction with FGF-1 and/or NRG-1 microparticles induced a significant improvement in the cardiac function as determined by echocardiography. These functional benefits were associated with a significantly reduced infarct size and fibrosis degree in comparison with the rats injected with non-loaded microparticles (control group).

7. Tissue revascularization was significantly increased in the groups treated with the FGF1-MP and NRG1-MP either combined or alone, which might have improved collateral flow and LV function.

8. A clear trend in the anti-apoptotic effect was detected after treatment with both cytokines at 90 days follow-up indicating the protective effect exercised by the cytokines.

9. c-Kit$^+$ progenitors were found in the lumen or periphery of the the blood vessels localized in the infarcted area of the hearts treated with FGF1/NRG1-MP. Importantly, this cell population was detected in higher levels than in the NL-MP control group, which might indicate that FGF-1 and NRG-1 cytokines released from the microparticles stimulate the recruitment of c-Kit$^+$ progenitors to the ischemic area.

10. Taken together, these findings demonstrate that the use of PLGA-MP delivery systems can significantly enhance the efficacy of the VEGF, FGF-1 and NRG-1 cytokines in infarcted hearts, paving the way for its therapeutic application in the setting of myocardial ischemia. Thus, local and sustained actions of these growth factors, released by PLGA-MP, trigger different mechanisms of cardiac repair and consequently promote cardiac regeneration.

CONCLUSIONES GENERALES

Las conclusiones de este trabajo son las siguientes:

1. Mediante la técnica del *Total Recirculation One-Machine System* (TROMS) se ha desarrollado una formulación basada en micropartículas de PLGA que es compatible con la administración intracardíaca en términos de tamaño de partícula, inyectabilidad y respuesta tisular.

2. Se ha encapsulado el $VEGF_{165}$ en micropartículas de PLGA. La proteína mantiene la actividad biológica tras su encapsulación ya que activa el receptor KDR en células endoteliales y, consecuentemente, induce proliferación celular.

3. En ratas infartadas tratadas con micropartículas cargadas con VEGF se ha observado la revascularización del tejido cardíaco, efecto no observado en ratas tratadas con la citoquina libre. Además, los vasos recién formados mostraban una estructura endotelial madura, lo que indica vascularización segura inducida por la liberación sostenida de la proteína a partir de las micropartículas. Asimismo, la estimulación tisular producida por el VEGF produce un remodelado positivo del corazón infartado.

4. Se ha observado que tanto el FGF-1 como la NRG-1 estimulan la proliferación de cardiomiocitos *in vitro*. Además, la combinación de estos dos factores redujo la apoptosis en cardiomiocitos adultos, lo que indica un efecto sinérgico entre ambas citoquinas.

5. Se han encapsulado las proteínas FGF-1 y NRG-1 en micropartículas de PLGA utilizando la técnica del TROMS. Los valores de eficiencia de encapsulación fueron elevados y ambas mantuvieron su bioactividad tras el proceso. Los ensayos de liberación *in vitro* mostraron que ambas citoquinas se liberan de forma sostenida.

6. El tratamiento del infarto agudo con micropartículas de FGF-1 y/o NRG-1 produjo, en un modelo murino, una mejora significativa de la función cardíaca. Estos beneficios funcionales se traducen en una disminución significativa en el tamaño del infarto y del nivel de fibrosis en comparación con los animales que recibieron micropartículas vacías (grupo control).

7. La revascularización del tejido cardíaco aumentó de forma significativa en los grupos tratados con micropartículas de FGF-1 y NRG-1 sólas o combinadas, lo que se tradujo en una mejora del flujo colateral y de la función del ventrículo izquierdo.

8. Noventa días después de la administración de las micropartículas cargadas con FGF-1 y NRG-1 se observó un efecto anti-apoptótico, debido a la acción protectora que ejercen las citoquinas.

9. En la zona infartada de los corazones tratados con la combinación de micropartículas de FGF-1 y NRG-1, se encontraron los progenitores c-Kit$^+$, tanto en el interior como alrededor de los vasos sanguineos. En los animales tratados se observaban mayores niveles en comparación con el grupo control, lo que podría indicar que las citoquinas liberadas de las micropartículas estimulan el reclutamiento de dichos progenitores hacia el área isquémica.

10. En conjunto, los resultados obtenidos en el modelo murino demuestran que el uso de los sistemas de liberación basados en micropartículas de PLGA mejoran significativamente la eficacia de VEGF, FGF-1 y NRG-1 en corazones infartados. La acción sostenida a nivel local de estos factores liberados desde las micropartículas activa diferentes mecanismos de reparación y, con ello, promueve la regeneración cardíaca, lo que abre camino hacia la aplicación de estas citoquinas en la terapéutica de la isquemia cardíaca.